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Characterisation of ALADIN's function during cell division

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Characterisation of ALADIN's function during cell division

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Supervisor Dr Eric Griffis

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Declaration

This thesis, submitted for the degree of Doctor in Philosophy at the University of Dundee, has been performed in the laboratory of Dr Eric Griffis at the Centre for Gene Regulation & Expression within the School of Life Sciences, Dundee. The presented work was performed under the guidance of Dr Eric Griffis, and contains no material that has been accepted for the award of any other degree in any university.

Sara Carvalhal

I declare that Sara Carvalhal has spent the equivalent of at least nine terms in the research department of the School of Life Sciences at the University of Dundee, and that she has fulfilled the conditions of Ordinance General No. 39 of the University of Dundee and is qualified to submit the accompanying thesis in application for the degree of Doctor of Philosophy.

Dr Eric Griffis

Supervisor

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Abstract

Cell division relies on many steps, precisely synchronised, to ensure the fidelity of chromosome segregation. To achieve such complex and multiple functions, cells have evolved mechanisms by which one protein can participate in numerous events on the cell life. Over the past few years, an increasing number of functions have been assigned to the proteins of the nuclear pore complex (NPC) also called nucleoporins. NPCs are large complexes studded in the nuclear envelope, which control the nucleocytoplasmic transport. It is now known that nucleoporins participate in spindle assembly, kinetochore organisation, spindle assembly checkpoint, and all processes important for genome integrity maintenance.

This work demonstrates that the nucleoporin ALADIN participates in mitosis, meiosis and in cilia.

In both mitosis and meiosis, ALADIN is important for proper spindle assembly. In mitosis, it was also discovered that ALADIN is a novel factor in the spatial regulation of the mitotic regulator Aurora A kinase. Without ALADIN, active Aurora A spreads from centrosomes onto spindle microtubules, which affects the distribution of a subset of microtubule regulators and slows spindle assembly and chromosome alignment. Interestingly, mutations in ALADIN causes triple A syndrome and some of the mitotic phenotypes observed after ALADIN depletion also occur in cells from triple A syndrome patients.

In meiosis, ALADIN contributes to trigger the resumption of meiosis in female mouse. Impairment of ALADIN from mouse oocyte slows spindle assembly, migration and reduces oocytes ability to extrude polar bodies during meiosis I, which concomitantly affects the robustness of oocyte maturation and impairs mouse embryo development.

Nucleoporins were also found at the base of the cilia, a centriole-derived organelle that participates in differentiation, migration, cell growth from development to adulthood. Here it is shown that ALADIN is also localised at the base of the cilia.

With this work, new ALADIN's functions have been identified across cell division, as well as uncovered an unexpected relation between triple A syndrome and cell division.

Dedication

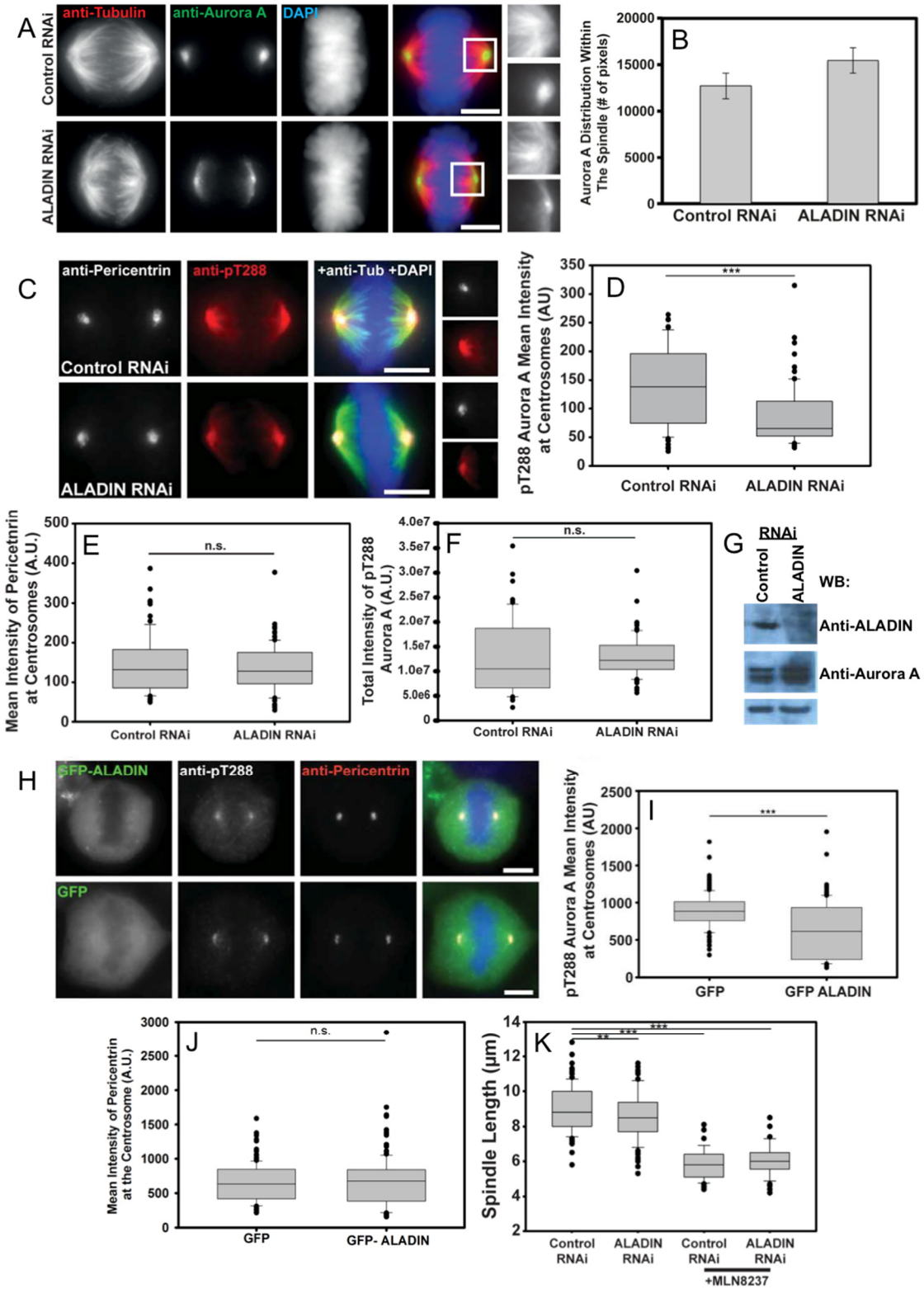
I dedicate this thesis to ALL of you for nursing me with affection and love and to make me believe that I can follow my dreams!!

A special thanks to my mentor Eric, my family, my friends and my love Daniel.

I am very grateful to have you ALL in my life, without you I could not make it!

Table of Contents

List of Figures and Tables



List of Abbreviations

ACTH – Adrenocorticotrophic hormone

ALADIN – Alacrima achalasia adrenal insufficiency neurologic disorder

AML – Myeloid leukemia

aMTOCs – acentriolar MTOCs

ANEA – Antinuclear envelope autoantibodies

APC/C – Anaphase promoting complex/ cyclosome

AU – Arbitrary units

AUL – Acute undifferentiated leukemia

BNIP3 – BCL2/adenovirus E1B 19kD-interacting protein 3

BSA – Bovine serum albumin

Cdk – Cyclin-dependent kinase

CEP192 – Centrosomal protein of 192 kDa

ch-TOG – Colonic and hepatic tumor over-expressed protein

ChIP – Chromatin immunoprecipitation

CML – Chronic myelogenous leukemia

CMML – Chronic myelomonocytic leukemia

DAPI – 4',6-diamidino-2-phenylindole

DDR – DNA damage response

DHCR24 –24-dehydrocholesterol reductase

DNA – Deoxyribonucleic acid

DSBs – DNA double strand breaks

dsRNAs – double stranded RNAs

DUSP1 – Dual specificity phosphatase 1

Eg5 – Thyroid receptor-interacting protein 5 also known as Kinesin-like protein 11

FG repeats – Phenylalanine-glycine repeats

FOXM1 – Forkhead box M1

FSH – Follicle stimulating hormone

G1 – First gap phase

G2 – Second gap phase

GDP – Guanosine diphosphate

GEF – Ran's guanine nucleotide exchange factor

GFP – Green fluorescent protein

GTP – Guanosine-5'-triphosphate

GV – Germinal vesicle

GVBD – Germinal vesicle break down

Haspin – Haploid germ-cell-specific nuclear protein kinase

HAUS – Augmin-like complex

HCG – Human chorionic gonadotropin

HEF1 – Adhesion-associated signaling protein

HR – Homologous recombination

HURP – Hepatoma upregulated protein

IF – Immunofluorescence

IU – International Units

IVF – *In Vitro* Fertilisation

JMML – Juvenile myelomonocytic leukemia

Kaps – Karyopherins

Kif2 – Kinesin Family Member 2

MAPs – Microtubules associated proteins

MCAK – Mitotic centromere-associated kinesin

MCAK – Mitotic centromere-associated kinesin

MCC – Mitotic checkpoint complex

MDS – Myelodysplastic syndrome

MLN8237 – Alisertib

MPF – Maturation promoting factor

Mps1 – Monopolar spindle-1

mRNA – Messenger RNA

mRNPs – Messenger ribonucleoparticles

MTOC(s) – Microtubule-organising centre(s)

Mtor – Megator

NDC1 – Nuclear Division Cycle 1 Homolog

NE – Nuclear envelope

NEBD – Nuclear envelope breakdown

NEDD1 – Neural precursor cell expressed, developmentally down-regulated 1

Neks – NIMA-related kinases

NESs – Nuclear export sequences

NHEJ – Non-homologous end joining

NIMA – Never-in-mitosis A

NLSs – Nuclear localisation sequences

NPC(s) – Nuclear pore complex(es)

NUDT1 – Nudix-type motif 1

NuMA – Nuclear mitotic apparatus protein

NUP(s) – Nucleoporin(s)

PA-GFP-tubulin – Photoactivatable-GFP-α-tubulin

PB – Polar body

PBC – Primary biliary cirrhosis

PCM – Pericentriolar matrix

Plk(s) – Polo-like kinases

PMSG – Pregnant mare serum gonadotropin

POMs – Pore membrane domain of the nuclear envelope

PTGS2 – Prostaglandin-endoperoxide synthase 2

PTMs – Post-translational modifications

RanGTP – GTPase Ran in the GTP-bound form

RCC1 – Regulator of chromatin condensation 1

RNA(s) – Ribonucleic acid(s)

RNAi – RNA interference

ROS – Reactive oxygen species

RT-PCR– reverse transcription PCR

S – Synthesis phase

SAC – Spindle assembly checkpoint

SAF(s) – spindle assembly factor(s)

CARA3 – Scavenger receptor class A, member 3

siRNAs – small interfering RNAs

STLC – S-Trityl-L-cysteine

T-ALL – T-cell acute lymphoblastic leukemia

Tacc3 – Transforming, Acidic Coiled-Coil Containing Protein 3

Tpr – Translocated promoter region

Tpx2 – targeting protein for Xklp2

UV – Ultraviolet

vs – versus

WB – Western blot

1.Introduction

Preamble

Actively dividing eukaryotic cells pass through a series of ordered events known collectively as the cell cycle, culminating, after cell growth and genome duplication, with division into two daughter cells. During these cycles of growth and division, cells are metabolically active but also dynamically reorganise their contents to ensure faithful chromosome segregation. To achieve such complex and multiple functions, cells have evolved mechanisms by which a same protein can participate at numerous events on the cell life. An example of this is the proteins of the nuclear pore complex (NPC) also called nucleoporins or NUPs, which have been found to have multiple roles in the cell. NPCs are large complexes studded in the nuclear envelope, which control the transport between the cytoplasm and nucleus and vice versa (Kahms et al., 2011). It is now accepted that NUPs have additional roles in contexts other than their traditional role in transport, and this has been the subject of interest in recent years. In particular, several NUPs have been described with important roles in regulating chromosome segregation during mitosis, reviewed in (Chatel and Fahrenkrog, 2011; Gigliotti et al., 1998; VanGompel et al., 2015). In this context, work in this thesis uncovered novel roles for the nucleoporin ALADIN in mitosis in somatic cells and meiosis in oocytes. This chapter contains an introduction to nucleoporins and cell division, with a special emphasis on the processes that regulates spindle assembly in mitosis and meiosis.

1.1.The nuclear pore complex and nucleoporins

The eukaryotic genome is sequestered within a double-layered nuclear membrane that separates this sensitive material away from the insults of cellular metabolism. However, packaging genetic material within a membranous boundary presents challenges for the cell, in particular (i) in controlling the flow of materials to and from the DNA, and (ii) the access for the segregation machinery to allow it to equally divided the duplicated genome during cell division. All eukaryotes evolved a common solution to the first problem. The nuclear envelope (NE) that consists of two lipid bilayers, the outer and inner nuclear membranes, is studded with nuclear pore complexes (NPCs) that allow for selective transport of import and export cargoes, and the diffusion of small molecules, reviewed in (Kahms et al., 2011).

The first structure of the NPCs was proposed in 1958, Wischnitzcr, S. et al 1958 in (Philipp Wagner, 1990), however the functional architecture of the NPC remains only partially understood (Hurt and Beck, 2015). Its overall structure is conserved among the species, with a core with octagonal rotational symmetry, made by eight identical spokes that form the central spoke-ring complex (Hoelz et al., 2011). At the centre of this structure, there is a central transport channel, where transport exchanges are performed. On each side of the NE lies one ring, facing the cytoplasm or the nucleoplasm (Hurt and Beck, 2015; Wente, 2000). Both outside rings are adorned. The cytoplasmic ring contains fibrils that are end-free and form a crown like structure. At the nucleus, nuclear fibrils lie in the nuclear ring and form a nuclear like structure, named nuclear basket (Strambio-De-Castillia et al., 2010).

On average, NPCs have an estimated molecular mass in vertebrates of 125 MDa, and they are composed of 30 different nucleoporins (Kahms et al., 2011). NPCs have very complex composition (Hurt and Beck, 2015; Mi et al., 2015) and a high degree of symmetry. Due to its size there is an inherent difficulty of counting proteins inside the single supramolecular complex (Mi et al., 2015), and some of its components have been shown to have functional redundancy. Both of these factors make the NPC's architectural organisation field controversial, reviewed in (Hurt and Beck, 2015). Most NUPs are named with the nomenclature "Nup" followed by their

molecular mass, which differs between species, and therefore there is not a uniform nomenclature of NUPs across species (Hoelz et al., 2011).

Computational approaches have shown that NUPs primarily consist of α-helical regions, β-propellers, and unstructured phenylalanine-glycine (FG) repeats (Devos et al., 2006; Wente and Rout, 2010). NUPs rich in FG repeats are called FG NUPs and represent nearly a third of all NUPs. These NUPs localise at the inner ring channel, where they participate directly at the nucleocytoplasmic transport (Strawn et al., 2004; Tran and Wente, 2006). However many FG domains were shown to not be essential to the transport *in vivo* (Zeitler and Weis, 2004).

According to a NUP's localisation within the NPC, they can be classified into six different categories: (a) integral membrane proteins of the pore membrane domain of the nuclear envelope (POMs), (b) outer ring nucleoporins, (c) inner ring nucleoporins, (d) linker nucleoporins (e) channel nucleoporins, also called central FG nucleoporins (f) nuclear basket nucleoporins, and (g) cytoplasmic filament nucleoporins, Figure 1 (Hoelz et al., 2011; Tran and Wente, 2006).

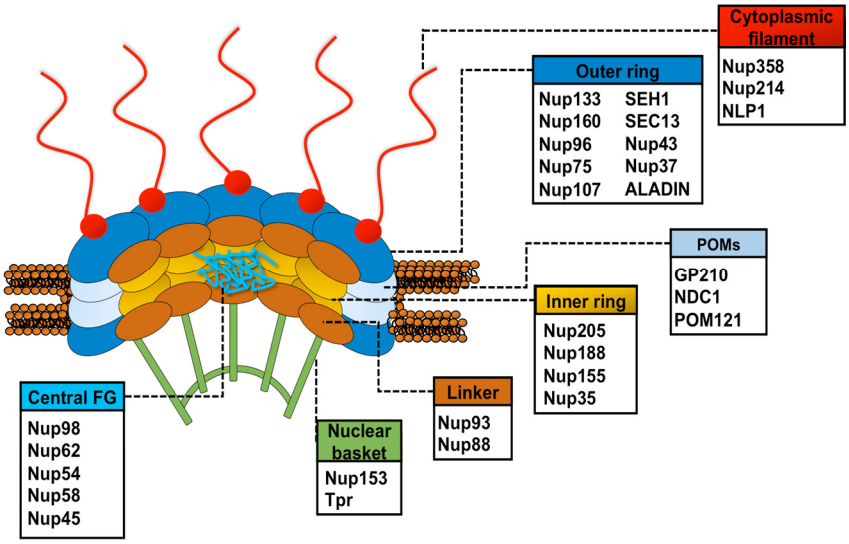


Figure 1: Scheme of mammalian nuclear pore complex (NPC) architecture.
The NPC essentially consists of a three ring-like structures, cytoplasmic ring (dark blue, cytoplasmic side), central ring (yellow) and nuclear ring (dark blue, nucleoplasmic side). Both outside rings are adorned with cytoplasmic filaments (red) and the nuclear basket (green). In vertebrate integral membrane proteins of the pore membrane domain of the nuclear envelope (POMs) are thought to anchor the NPC to the NE. Multi-copies of approximately 30 different NUPs compose NPC, which can be classified into different categories according with their structure and/or localisation.

1.2.Nucleocytoplasmic transport

The NE sequesters the genetic material from the surrounding cytoplasm. Exchanges of molecules between the two compartments completely rely on NPCs (Wente, 2000). While NPC allows free diffusion of water, ions, small metabolites, and small proteins; it is a highly selective transporter for macromolecules greater than 40 kDa (Wente and Rout, 2010). These macromolecules include proteins, RNAs, ribosomal subunits, and viral particles, and they are common termed cargos (Macara, 2001). At the centre of the NPC channel there are FG NUPs, which resemble a portal due to their extended unfolded structures, which all interact in the central pore. FG NUPs work as a selective barrier, and they also coordinate the nucleocytoplasmic transport, allowing the docking of transport receptors (Hoelz et al., 2011). Karyopherins (hereinafter referred to as kaps) were the first family of transport receptors discovered (Melchior et al., 1995b). They are called importins when they mediate the nuclear import of macromolecular cargos, whereas those mediating the export of cargos to the cytoplasm are generally known as exportins (Philipp Wagner, 1990). All the information required to target macromolecules to the nucleus or cytoplasm is within specific amino acid sequence motifs termed nuclear localisation sequences (NLSs) or nuclear export sequences (NESs), respectively, reviewed in (Hanover, 1992). The full spectrum of these sequences and their regulation and recognition by each transport receptor has not yet been fully defined (Marfori et al., 2011). These cargo molecules can directly interact with β-karyopherins (β-kaps), or indirectly via an adaptor karyopherin (α-kap). Binding to an α-kap drives a conformational change that then allows the α-kap to bind to a β-kap. Once the cargo is associated with β-kaps, they dock at FG NUPs sites at one side of the NE. After this, the cargo is translocated to the opposite side and released. The transport directionality is driven by the GTPase Ran (Hoelz et al., 2011). Ran can adopt two distinct conformations, depending on the bound nucleotide (GTP, higher concentrated in the nucleus or GDP, mainly at the cytoplasm). In the case of importins, they release their cargos when they bind to RanGTP. On the other hand, exportins associate with cargos and RanGTP. Their cargos are released when a RanGEF exchanges GTP, which localises on the cytoplasmic side of NPC (Cook et al., 2007; Melchior et al., 1995a).

1.3.Nucleoporins and unconventional functions

Besides NUP's role in nucleocytoplasmic transport, several NUPs have been implicated in other cellular processes including transcriptional regulation, DNA damage repair, chromosome segregation and cilia function (Figure 2) (Franks and Hetzer, 2013; Light and Brickner, 2013; Mendjan et al., 2006).

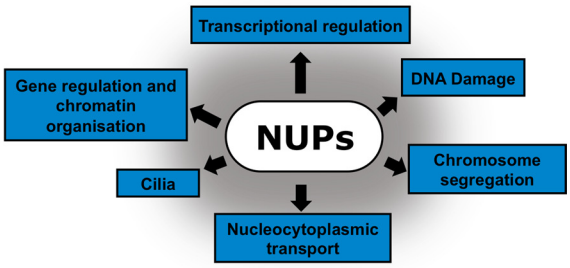


Figure 2: Functional roles of NUPs.
Schematic diagram representing NUPs role described in the literature, showing their participation in multiple cellular processes.

1.3.1.Transcriptional regulation

In eukaryotic gene expression, mRNA synthesis and processing occurs in the nucleus and translation happens in the cytoplasm (Bonnet and Palancade, 2014). To facilitate mRNA export through NPCs, mRNAs are assembled into messenger ribonucleoparticles (mRNPs). The movement of properly processed and assembled mRNPs from the actively transcribing genes to the cytoplasm is critical, and therefore subject to quality control by a nuclear mRNP surveillance mechanism (Bjork and Wieslander, 2014). The direct participation of NUPs in mRNP export is controversial. Albeit some studies have shown that mRNP export can be modulated by NUPs, reviewed in (Culjkovic-Kraljacic and Borden, 2013); *in vivo* mRNA export kinetics studies have revealed that inhibition of mRNA-NPC interactions do not affect nucleoplasmic and cytoplasmic mRNP dynamics, indicating that direct interaction of mRNPs with NUPs is not essential export (Mor et al., 2010).

Nonetheless, live dynamic studies in mammalian cells have showed that mRNPs migrate between compact chromatin domains and the NPC inner channel several times before being transport (Mor et al., 2010). These results suggest the participation of NPC in the quality control of nuclear mRNPs (Mor et al., 2010). In agreement with this idea, it is known that the Swt1 RNA

endonuclease, which participates in quality control of nuclear mRNPs in yeast, is associated with the nuclear pore complex (Skruzny et al., 2009). Further work is necessary to better understand the role of NPCs and their NUPs on the mRNA export pathway and quality control.

1.3.2. Gene regulation and chromatin organisation

Nucleoporin chromatin immunoprecipitation (ChIP) throughout the genome uncovered that NUPs can bind to the DNA in particular regions with high transcriptional activity (Casolari et al., 2005; Casolari et al., 2004; Kalverda and Fornerod, 2010). Nup98 is recruited to the chromatin where it regulates the transcription of Trx target genes (Hox genes) involved in *Drosophila* development (Pascual-Garcia et al., 2014).

Transcriptional memory is the process by which previously expressed genes are frequently primed for re-activation. Experiments performed in yeast NPC have shown that this mechanism requires changes in chromatin structure and a physical interaction with NUPs, namely Nup98 and Nup100, reviewed in (D'Urso and Brickner, 2014).

NUPs can also change chromatin organisation (Ibarra and Hetzer, 2015). Chromatin usually occupies the entire nucleus, but in oocytes meiotic chromatin becomes completely detached from the NE and forms a more condensed chromatin filament structure (karyosome). Depletion of Nup62 or Nup93 in *Drosophila* oocytes disrupts the compact karyosome morphology. This mechanism is conserved in somatic cells, showing that a negative loop within the nuclear pore complex controls global chromatin organisation (Breuer and Ohkura, 2015). In yeast, it was also observed that interaction between INO1 gene and Nup100 changes the chromatin structure and rates of transcriptional reactivation rates (Light et al., 2010). All these evidence demonstrate that NUPs can actively participate in chromatin reorganisation and transcription, giving an extra layer of complexity in the structure and regulation of NPCs.

1.3.3. DNA Damage

DNA damage result from many endogenous and environmental agents, and such lesions can promote genomic instability and cell death. These DNA lesions can break a strand of DNA or remove or change a base from the backbone of DNA. Their repair and protection depends on large

machinery (Jackson and Bartek, 2009). One of them is the DNA damage response (DDR), which mainly act through be repair by homology-based repair (homologous recombination (HR)) or direct joining across the break site (non-homologous end joining (NHEJ)) (Jackson and Bartek, 2009).

Nup153 gives one of the stronger pieces of evidences that NUPs have roles in protecting against DNA damage. Impairment of human Nup153 delays DSB repair and impairs cell survival (Moudry et al., 2012). It is now known that Nup153 mediates the nuclear import of 53BP15, a major mediator in the repair of DNA double strand breaks (DSBs) (Lemaitre et al., 2012; Moudry et al., 2012). Also, Nup153 regulates the choice between the NHEJ and HR pathways via recruitment of 53BP15 (Lemaitre et al., 2012). HR pathways in yeast are also dependent on Nup84 (Loeillet et al., 2005) and Nup60 (Palancade et al., 2007). Furthermore, yeast mutants of the Nup133 and Nup120 are highly sensitive to DNA-damaging treatments (Loeillet et al., 2005), suggesting a role of these NUPs in DNA damage responses. Interestingly, human Nup107 has also been associated with the DNA damage-dependent nuclear accumulation of Apaf-1, which is important for DNA damage-induced intra-S phase checkpoint response (Jagot-Lacoussiere et al., 2015).

Oxidative stress is defined as an excessive production of reactive oxygenated species (ROS) that cannot be counteracted by the action of antioxidants. As a result of cell redox unbalance, ROS can induce modifications on the protein structure and/or possibility modulate their functions, such as DNA lesions, reviewed in (Barzilai and Yamamoto, 2004). Mutations or depletion of the NUP ALADIN causes hypersensitivity to oxidative stress (Juhlen et al., 2015; Kind, 2010). The function of this NUP in oxidative stress is further explored in the section 1.4.5.2.ALADIN.

1.3.4.Chromosome segregation

For successful genome segregation, chromosomes are divided into two daughter cells by the mechanical activity of the microtubule spindle, which binds chromosomes. In yeast, the NE remains intact throughout the cell cycle. The spindle pole bodies are embedded in the NE, and kinetochores remain attached to spindles for most of the cell cycle. Other eukaryotic kingdoms

have open and semi-open mitoses where the NE is ruptured to some degree during early stages of mitosis and reassembles after anaphase onset (De Souza and Osmani, 2007). In open mitoses, after the NE breaks down, the process of nuclear transport is no longer operating and NPCs are partially disassembled. At that point, many NUPs gain new functions to facilitate spindle assembly and/or post-mitotic nuclear formation (Chatel and Fahrenkrog, 2011) (Figure 3).

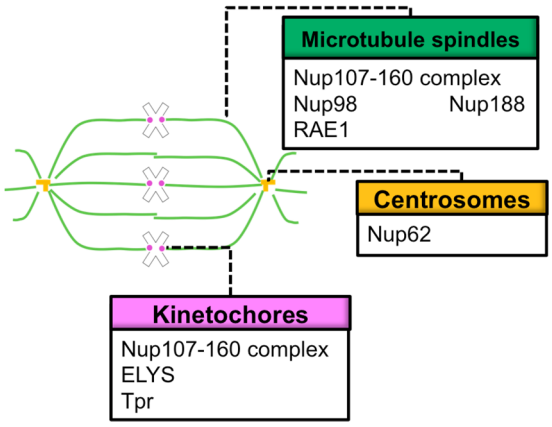


Figure 3: Schematic diagram of NUPs localisation within the mitotic spindle.
The Nup107–160 complex and RNA export 1 (RAE1)-containing ribonucleoproteins (RNPs) localise to spindle microtubules while if in conjunction with its chromatin-targeting factor ELYS is recruited to kinetochores. Nup98 and Nup188 are required for bipolar spindle formation and Nup62, which localises at the centrosome during metaphase, is important to maintain centrosome homeostasis.

The first evidence that NUPs could have roles in mitosis came from the observation that a conserved NPC subcomplex (Nup133 and Nup84/Nup107) was associated with kinetochores during mitosis (Belgareh et al., 2001). Subsequent studies showed the localisation of the Nup107-160 complex at the kinetochores (Loiodice et al., 2004), together with another Nup called ELYS (Rasala et al., 2006; Rasala et al., 2008). In addition, the Nup107-160 complex can also localise to the mitotic spindle. This complex has been shown to participate in chromosome alignment and segregation (Platani et al., 2009; Prunuske et al., 2006). Other NUP involved in chromosome aligment is Tpr, a NUP at the nuclear basket (Bangs et al., 1996). Recently Tpr was recognised as a novel Mad2-interacting protein, and participates in the spindle assembly checkpoint signalling (Lee et al., 2008). Nup98 was shown to regulate bipolar spindle assembly through its association with microtubules and by opposition of the microtubule depolymerase MCAK (Cross and Powers, 2011). Nup98 directly binds to RAE1 (which is structurally similar to Bub3), and $RAE1^{-/-}$ knockout mice exhibit mitotic checkpoint defect and chromosome mis-segregation, suggesting also RAE1's participation in cell division functions (Babu et al., 2003). In Nup188-

depleted mitotic cells, chromosomes fail to align on the metaphase plate, and the microtubule bundles that attach to kinetochores are severely weakened (Itoh et al., 2013). Recently, Nup62 was identified at the centrosome. The centrosome is a small organelle capable of duplicating itself once per cell cycle under normal conditions; but if Nup62 is absent from human cells, aberrant centrosome formation is observed together with mitotic cell death (Hashizume et al., 2013). Finally, RanBP2/Nup358 was shown to be a regulator of the nuclear envelope breakdown and formation (Prunuske et al., 2006) and GTPase Ran a major regulator of mitotic spindle assembly and maintenance (detailed in 1.8.Spindle assembly) (Kalab and Heald, 2008).

1.3.5.Cilia

Primary cilia are vital coordinators of many signalling pathways from development to tissue homeostasis. Due to a wide variety of ciliary functions, defects in ciliar structure and function are linked to a multiplicity of human diseases and developmental disorders. Recently several NUPs have been found at the base of primary cilia (Kee et al., 2012; Kee and Verhey, 2013). Although Nup358, Nup214, Nup62 and Nup153 (FG NUPs) were localised at the base of the cilia, POMs NUPs were not. Similarly with their role at the NPC, it was proposed that these NUPs form a selective barrier-exclusion at the base of the cilia where it concentrates proteins targeted to the cilia, and excludes others (Kee et al., 2012; Kee and Verhey, 2013; Obado and Rout, 2012).

1.4.Nuclear pore proteins and diseases

In the previous section, it was covered the roles of NUPs in non-transport activities in different cellular behaviours, as well as in other events. Therefore, mal-function of these proteins can be associated with several diseases (Cronshaw and Matunis, 2004).

1.4.1.Autoimmune disorders

1.4.1.1.Primary biliary cirrhosis

Primary biliary cirrhosis (PBC) is characterised by features of autoimmune hepatitis that slowly destroys the bile ducts, which eventually causes liver cirrhosis (Wesierska-Gadek et al., 2007). Approximately 50% of these patients' serum has antinuclear envelope autoantibodies (ANEA, which recognise the nuclear ring of the NPC) (Ruffatti et al., 1985; Wesierska-Gadek et al., 2007), including autoantibodies against gp210 and Nup62 (Duarte-Rey et al., 2012; Miyachi et al., 1996). To date, it is still unclear how or whether gp210 and nup62 are related with the pathological phenotype (Worman, 2007). However their autoantibodies levels were correlated with higher risk of end-stage of hepatic failure (Nakamura et al., 2006). It was suggested that increased expression of gp210 in small bile ducts is probably associated with damage of biliary epithelial cells by inflammation; possibly caused by the autoimmune response to gp210, which eventually leads to the progression of end-stage hepatic failure in PBC (Nakamura et al., 2006).

1.4.1.2.Other immune diseases

PBC is the most characterised immune disease related to nuclear pore proteins. Nonetheless, reactivity against other NUPs have also been found in several autoimmune disorders. RanBP2/Nup358, Nup98, Nup62 and gp210 were recognised in serum from patients with autoimmune myositis (Senecal et al., 2014). Nup62 autoantibodies were also found in serum from patients with systemic lupus erythematosus (Kraemer and Tony, 2010). Autoantibodies against Nup153 and Tpr have been correlated with autoimmune liver diseases and systemic rheumatic diseases (Enarson et al., 2004).

1.4.2.Cancer

Translocated Promoter Region (Tpr) was the first link between tumorigenesis and NPC components (Cooper et al., 1984). This protein was discovered as a product of a chromosomal translocation fusion with tyrosine kinase Hepatocyte Growth Factor Receptor (Cooper et al., 1984). Following studies identified Tpr as a component of the nuclear basket (Bangs et al., 1996). Since then, others NUPs have been associated with cancer either by their elevated expression in tumours (Nup88), or through chromosomal translocations (Nup62, Nup98, Nup214, RanBP2/Nup358), reviewed in (Simon and Rout, 2014). Overexpression of Nup88 has been found in ovary, stomach, colon, liver, prostate, and lung cancers. Its mRNA overexpression was associated with high aggressiveness of breast cancer (Agudo et al., 2004; Martinez et al., 1999; Simon and Rout, 2014; Zhao et al., 2010). The link between this FG NUP with tumorigenesis is still poorly understood, but it was suggested to be related with mRNA export together with Nup214 and cell division. Furthemore, when this NUP is impaired there is a delayed degradation of mitotic marker proteins cyclin B1 and A (Bernad et al., 2006; Bhattacharjya et al., 2015; Hashizume et al., 2010; Hutten and Kehlenbach, 2006). Nevertheless, Nup214 can also suffer chromosomal translocations, producing oncogenic fusion proteins (Simon and Rout, 2014).

Fusion proteins containing Nup214 were identified in patients diagnosed with acute myeloid leukemia (AML), acute undifferentiated leukemia (AUL), myelodysplastic syndrome (MDS), and T-cell acute lymphoblastic leukemia (T-ALL) (Kohler and Hurt, 2010). Distinct Nup98 fusion proteins have also been linked to cancer such as AML, MDS and T-ALL. Additionally, it was also associated with chronic myelogenous leukemia (CML), chronic myelomonocytic leukemia (CMML), and juvenile myelomonocytic leukemia (JMML), deeply reviewed in (Gough et al., 2011). The exact molecular mechanism by which Nup98 participates in tumorigenesis remains unknown (Gough et al., 2011; Wang et al., 2007). Nonetheless, this NUP participates in numerous functions as described above and their fusion with other proteins can impair NUP98's role.

1.4.3.Cardiovascular diseases

Several NUPs have been associated with ischaemic and dilated heart disease in humans, as it has been shown that increased levels of NDC1, Nup160, Nup153 and Nup93 were associated with

heart failure in human cardiomyocytes (Tarazon et al., 2012). Moreover, during hypertrophy, both rat and human cardiomyocytes present changes of Nup62 levels, and in factors involved in the nucleocytoplasmic transport (RanBP1, α and β importins) (Chahine et al., 2015).

The homozygous mutation, R391H in the nucleoporin Nup155 affects its nuclear localisation and reduces nuclear envelope permeability (Zhang et al., 2008). This mutation also leads to atrial fibrillation and early sudden cardiac death. This strongly suggests a role of Nup155 in cardiovascular diseases (Zhang et al., 2008).

1.4.4. Viral infections

The NPC acts as a selective barrier mediating the transport of RNA and proteins between the nucleus and the cytoplasm, presenting a challenge to viral pathogens. However, viruses have evolved specific mechanisms to modulate its permeability, in particular by targeting the NPC (Cohen et al., 2012). A deep review of how each type of virus regulates and ultimately take over the host functions can be found here (Le Sage and Mouland, 2013). For example, large viruses with nucleocapsids are not physically able to pass through the central channel of NPCs. In the case of human adenoviruses (Ad2 and Ad5), their capsids bind to the NPC filament proteins, in particular to Nup214 and RanBP2/Nup358. These interactions disrupt NPC and consequently NE increases permeability granting the viral genome access to the nucleus (Strunze et al., 2011). Another way to disrupt NPC integrity used by human rhinovirus (HRV) is through the cleavage of NUPs, namely Nup62 (2 h post-infection), Nup98 (4.5 h post-infection), and Nup153 (6 h post-infection) (Watters and Palmenberg, 2011). Moreover, BGLF4 of Epstein-Barr virus (Verlhac et al.) encodes a serine/threonine protein kinase that presumably phosphorylates Nup62 and Nup153, which disrupts the NPC permeability barrier to allow the virus to enter the nucleus (Chang et al., 2012).

1.4.5. CNS disorders

Triple A syndrome was the first described hereditary neurodegenerative disorder caused by mutations in a NUP. However three other NUPs have been associated with central nervous system disorders (Nup62, Nup155 and RanBP2/Nup358). In particular, a missense mutation (Q391P) in

Nup62 was found in patients with autosomal recessive infantile bilateral striatal necrosis (Basel-Vanagaite et al., 2006). A mutation found in Nup155 was shown to cause atrial fibrillation and early sudden cardiac death (Zhang et al., 2008). Additionally, acute necrotizing encephalopathy seems to be caused by mutations in the nuclear pore component RanBP2/Nup358 (Neilson et al., 2009). The precise mechanisms between these diseases and their correspondent mutated NUPs are still unknown.

1.4.5.1.Triple A syndrome

Triple A syndrome is characterised by a triad of adrenocorticotrophic hormone (ACTH)-resistant adrenal insufficiency, alacrimia and achalasia cardia. Allgrove and his colleagues in 1978 were the first to described two unrelated pairs of siblings, where all four suffered from ACTH insensitivity and achalasia, three had alacrimia and one had autonomic dysfunction. (Allgrove et al., 1978). Since then, approximately 100 cases have been reported in the literature (Misgar et al., 2015), showing that triple A is a rare autosomal recessive multi-system disorder. It is frequently associated with gradual autonomic disturbances (abnormal pupillary reflexes, poor heart rate variability and orthostatic hypotension), progressive neurological degeneration (cerebellar ataxia, and peripheral neuropathy), and others neurological manifestations such as polyneuropathy, deafness and mental retardation (Misgar et al., 2015). It has also been associated with more rare symptoms such as low bone mineral density (Dumic et al., 2015), delayed puberty (Bustanji et al., 2015) and aldosterone deficiency (Huebner et al., 1999). Triple A syndrome manifests its first features during the first decade of life of patients, achalasia and alacrimia being the most valuable clinical signs to reach the diagnosis. The primary cause of mortality in this syndrome is adrenal crisis with glucocorticoid deficiency, which is usually treated with glucocorticoid and if necessary mineralocorticoid replacement. Also, alacrimia is treated with artificial tears and achalasia cardia can be treated with pneumatic dilatation (Sarathi and Shah, 2010).

Triple A syndrome was formerly known as Allgrove syndrome and later named “4A” syndrome, although “triple A” is the most known and used nomenclature (Cronshaw and Matunis, 2004).

In 2000, three independent reports described a novel gene (AAAS) in chromosome 12q13 encoding a protein of 547 amino acids, and its mutant form was found in individuals with triple A syndrome (Huebner et al., 2000; Lee et al., 2000; Tullio-Pelet et al., 2000). Homozygous truncating mutations were found in unrelated patients indicating that the predicted product of AAAS, ALADIN (for alacrima-achalasia-adrenal insufficiency neurologic disorder) was involved in triple A syndrome (Huebner et al., 2000; Lee et al., 2000; Tullio-Pelet et al., 2000). Two years later, ALADIN was identified as a NUP in a proteomic analysis of the mammalian nuclear pore complex (Cronshaw et al., 2002). ALADIN was the first NUP linked to a human inheritable disease (Prasad et al., 2014).

1.4.5.2. ALADIN

The majority of the studies of ALADIN function have been made in the context of this disease. The ALADIN protein and its correspondent mRNA (AAAs mRNA) are ubiquitously expressed with a predominance in adrenal cells and in central nervous system structures in human and rat (Cho et al., 2009; Storr et al., 2005). Structurally, ALADIN contains seven WD repeats in its central region. These domains are most abundant in eukaryotes and are characterised by the presence of dipeptides constituted of tryptophan (W) and aspartate (D) (Tullio-Pelet et al., 2000). Although it is known that proteins with these repeat domains regulate several biological processes such as signal transduction, gene expression regulation, genome stability and cell cycle control (Xu and Min, 2011; Zhang and Zhang, 2015), the role of these repeats in ALADIN remains unclear.

Microscopy images revealed that GFP-ALADIN is localised in the cytoplasmic face of NPCs (Cronshaw and Matunis, 2003). ALADIN's NPC localisation requires its C-terminus domain and its interaction with NDC1 (Nuclear Division Cycle 1), an integral membrane at the NE (POMs) (Cronshaw and Matunis, 2003; Kind et al., 2009; Yamazumi et al., 2009). NDC1 forms a link between the NE membrane and soluble nucleoporins, and it may interact with ALADIN through its C-terminus region, which is exposed to the cytoplasm (Mansfeld et al., 2006). The depletion of NCD1 abolishes ALADIN's localisation at the NPC, however ALADIN also mediates NDC1 localisation at the NPC (Cronshaw and Matunis, 2003; Kind et al., 2009).

Immunofluorescence techniques revealed that several mutations described in triple-A syndrome can mis-localise ALADIN from the NE, but not other NUPs (Cronshaw and Matunis, 2004; Huebner et al., 2004). To date, several mutations have been described (Figure 4). Although it may help in the diagnosis of this syndrome, based on DNA analysis the clinical outcome of the disorder cannot be predicted by the expression of the mutant protein form; patients with the same mutation have variable phenotypes (Huebner et al., 2004; Milenkovic et al., 2008).

Fibroblast cells from patients have been used to better understand the molecular mechanisms of triple A syndrome. (Hirano et al., 2006; Kind, 2010; Koehler et al., 2013). Recently, ALADIN loss-of-function studies were performed in adrenocortical and neuronal cells, which are the most commonly affected in triple A syndrome (Juhlen et al., 2015; Prasad et al., 2013). In an attempt to have a mouse model to study this syndrome, mice lacking ALADIN or expressing two major mutations found in this syndrome (p.Q15K and p.S263P) were produced (Huebner et al., 2006 and Huebner personal communication). However, they did fail to develop a phenotype resembling human triple A syndrome, even though this protein is highly conserved in both species. Nevertheless, female infertility was observed in mice lacking ALADIN or expressing p.Q15K ALADIN, a mutation that generates a truncated protein that only contains the first 15 amino acids (Huebner et al., 2006 and Huebner personal communication).

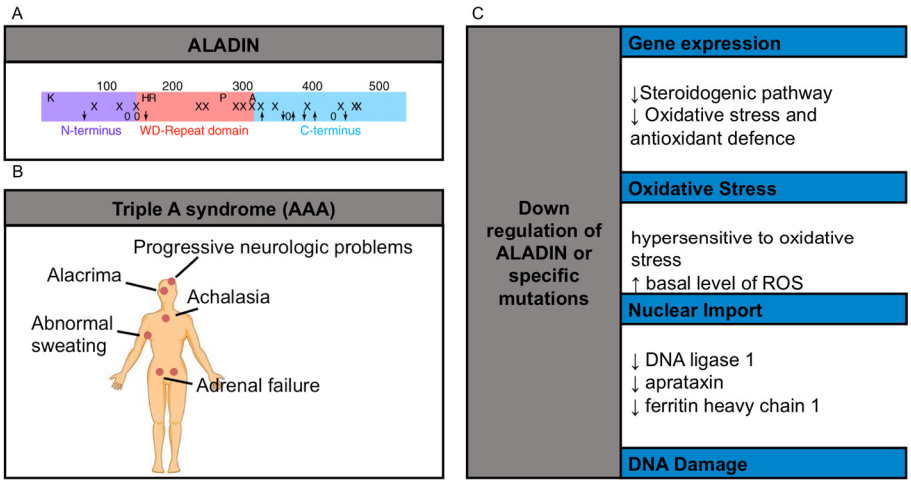


Figure 4: The protein ALADIN and its associated disease.
(A) Domain structure of the ALADIN protein. The central WD-repeat domain (red) is flanked by the N- and C-terminus domains (purple and blue, respectively). Mutations associated with triple A syndrome are represented. Letters represent substitution caused by missense mutation; X, premature stop caused by a nonsense mutation; ↑, frameshift (insertion); ↓, frameshift (deletion); 0, splice site mutation. (B) Triple A syndrome is highly variable, even among related patients. One of the most consistent symptoms of triple A syndrome is a defect in tear production (alacrima), along

with adrenal failure and achalasia. Most triple A patients have some form of neurological problems and often abnormal sweating. Adapt from (Cronshaw and Matunis, 2004) (C) Schematic diagram representing defects observed after down regulation of ALADIN or in mutations in ALADIN described in triple A patients.

ALADIN's role at the NPC is poorly understood (Prasad et al., 2014), however it has been implicated in the import of different factors into the nucleus, which prevents the cell against DNA damage and subsequent cell death when subjected to oxidative stress (Hirano et al., 2006; Juhlen et al., 2015; Kind et al., 2009; Prasad et al., 2013; Yamazumi et al., 2009). In particular, expression of the I482S ALADIN mutant form, found in triple A syndrome, or downregulation of ALADIN in adrenocortical or neuronal cells caused hypersensitive to oxidative stress (Hirano et al., 2006; Juhlen et al., 2015; Prasad et al., 2013). These alterations of ALADIN impaired nuclear import of DNA ligase 1, aprataxin (a repair protein required for repair of DNA single-strand breaks), and ferritin heavy chain 1 (Hirano et al., 2006; Juhlen et al., 2015; Prasad et al., 2013). Primary cells of patient with triple A (p.S263P, p.Q387X, p.R342X, p.H160R, IVS14G>A, p.W84X, p.W295X, p.W295X, IVS14G>C, p.W295X, IVS14G>A, p.R119X/K301N, p.W84X/L430F) showed a 2.1-fold increased basal level of reactive oxygen species (ROS), which was massively boosted after induction of artificial oxidative stress by paraquat (Kind, 2010). Moreover, the expression of target genes associated with oxidative stress and antioxidant defences was analysed in fibroblast cultures of triple A syndrome cells (Koehler et al., 2013). This analysis showed a significant and differential regulation of important genes in the process of reactive oxygen homeostasis, namely BCL2/adenovirus E1B 19kD-interacting protein 3 (BNIP3), 24-dehydrocholesterol reductase (DHCR24), dual specificity phosphatase 1 (DUSP1), forkhead box M1 (FOXM1), nudix-type motif 1 (NUDT1), prostaglandin-endoperoxide synthase 2 (PTGS2), and scavenger receptor class A, member 3 (SCARA3) (Koehler et al., 2013).

Also, AAAs depletion in adrenal cells reduced expression of key components of the steroidogenic pathway, including genes coding for cytochrome P450 hydroxylases (CYP17A1 and CYP21A2), cytochrome P450 oxidoreductase, and Steroid 11β-Hydroxylase (P450c11β) providing further compelling evidence that ALADIN is directly related with the adrenal insufficiency described in the triple A syndrome (Juhlen et al., 2015; Prasad et al., 2013).

The detailed mechanism by which redox balance is maintained through ALADIN is still unclear. Nonetheless, any perturbation in this balance, such as an increase in reactive oxygen species (ROS), could lead to oxidative stress and may lead as well to cell dysfunction/damage/death and DNA damage (Prasad et al., 2014). Moreover, steroidogenesis significantly contributes to mitochondrial ROS production. On the other hand, ROS can obstruct steroidogenesis (Prasad et al., 2014). To date, there is a lack of detailed information to identify the role of ALADIN in these processes and in the pathogenetic mechanisms of triple A syndrome (Figure 4) (Prasad et al., 2014; Sarathi and Shah, 2010).

In this thesis it is described that ALADIN also participates in mitosis, meiosis and cilia. Thus, in the next section an overall description of mitosis and its regulation is presented.

1.5.Cell cycle

Most eukaryotes follow a process of growth and division termed cell cycle. The cell cycle is a complex process that involves an ordered set of events controlled by numerous regulatory proteins that take the cell through the production of two daughter cells. Throughout interphase, the larger phase of the cell cycle, the cell is engaged in metabolic activities. This stage can be sub-divided into three phases: first gap phase (G1), synthesis phase (S), and second gap phase (G2). In addition, cells can become quiescent by entering into a G0 phase, a separated stage from interphase, Figure 5. During Go, cells can assemble cilia, sensory organelles that are invariably resorbed before mitotic entry (M stage) (Plotnikova et al., 2009).

In G1, the cell grows and increases in size to ensure that all the machinery is ready for DNA replication, which happens in S phase. After DNA duplication, the cell continues to grow in order to have all the mechanisms ready for cell division. In mitosis replicated chromosomes must be equally and faithfully segregated into the two identical cells.

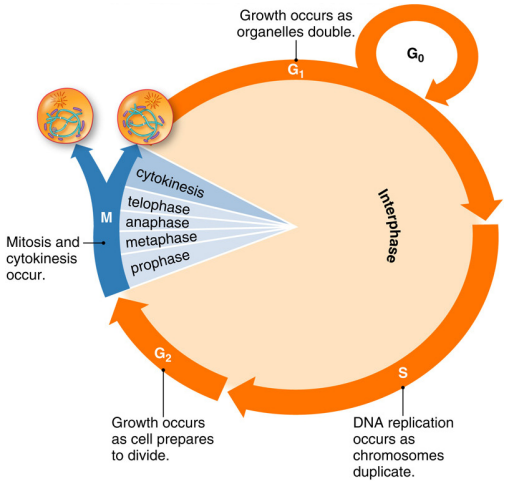


Figure 5: Cell cycle.
The cell cycle is categorised by four distinct phases: the first gap phase (G1); the DNA synthesis phase (S); the second gap phase (G2); and finally mitosis (M). Quiescent cells that have 'dropped out' of the cell cycle are in G0 phase. G1, S and G2 occur during interphase (Allan et al.), which is followed by mitosis (blue). Cilia can be assembled during G1/G0. From Perkepi (http://www.perkepi.com/cell-cycle/).

1.6.Mitosis

After DNA has been replicated properly, each chromosome is formed of two sister chromatids, that are linked by cohesion rings (Kschonsak and Haering, 2015). Prior to the start of mitosis, chromosomes are condensed in preparation for division. Mitosis was first described by Fleming and it was divided into five events: prophase, prometaphase, metaphase, anaphase and telophase/cytokinesis (Figure 6), and it culminates in the formation of two daughter cells (Fleming, 1882) reviewed in (Civelekoglu-Scholey and Scholey, 2010).

During prophase, just after nuclear envelope breakdown (NEB), microtubules emanating from duplicated centrosomes capture sister chromatids by attaching to their kinetochores, the specialised protein rich platforms assembled upon centromeric DNA. Chromosomes become rapidly bi-oriented, moving towards the centre of the cell. This process is called chromosome congression and when it is completed and all chromosomes have reached what is called the metaphase plate, cells have reached metaphase. At the metaphase, chromosomes align at the middle of the spindle, the metaphase plate. Once chromosomes are properly aligned and the spindle assembly checkpoint (SAC) is satisfied, the anaphase promoting complex/cyclosome (APC/C) mediates ubiquitin-dependent degradation of the separase inhibitor securin and cyclin B, which induces the removal of cohesin from sister chromatids and drives cells out of the mitotic state, respectively. APC/C activation results in anaphase onset and the movement of separated sister chromatids to the opposite spindle poles. The cell division plane is determined by spindle-cortex interactions, and a contractile actin-myosin ring forms a cleavage furrow between the segregated DNA masses in a process called cytokinesis. During telophase, the chromosomes begin to decondense and the NE reassembles, forming two daughter nuclei. At the same time, cytokinesis is completed by abscission of the cleavage furrow, resulting in two nascent daughter cells (Pines and Rieder, 2001).

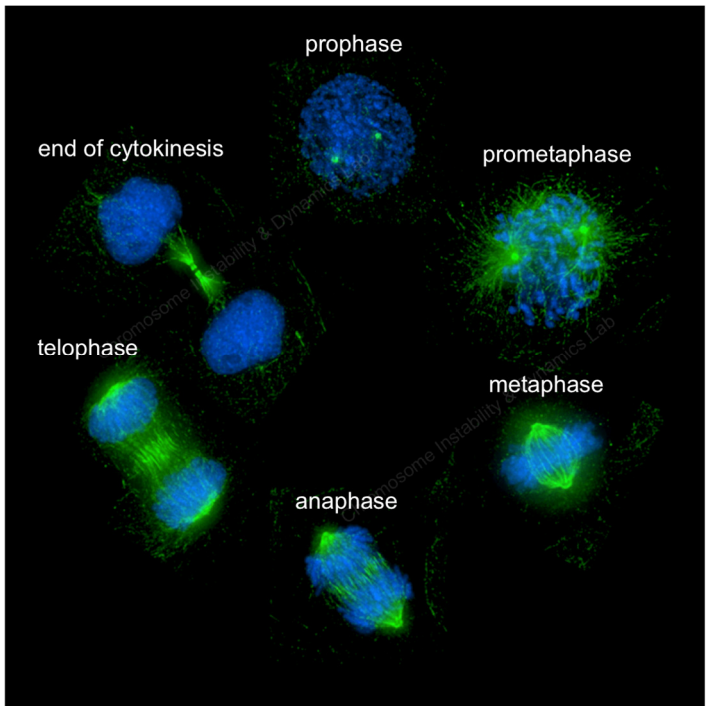


Figure 6: Events of mitosis.
Immunofluorescence images of mitotic cells, where tubulin is showed in green and DNA in blue. Mitosis is subcategorised into five stages: prophase, prometaphase, metaphase, anaphase and telophase/cytokinesis. When mitosis is complete, two identical daughter cells are generated. Adapted from the Chromosome Instability & Dynamics Lab (http://www.ibmc.up.pt/cid).

1.7.The mitotic spindle and its components

The mitotic spindle is the major machine that powers mitosis (Pines and Rieder, 2001). The spindle is composed by an array of microtubules with aligned chromosomes in the middle of the metaphase plate (Figure 7). In the majority of higher eukaryotes, each pole contains a centrosome, an organelle structurally composed by a pair of centrioles surrounded by the pericentriolar matrix (PCM) (Civelekoglu-Scholey and Scholey, 2010).

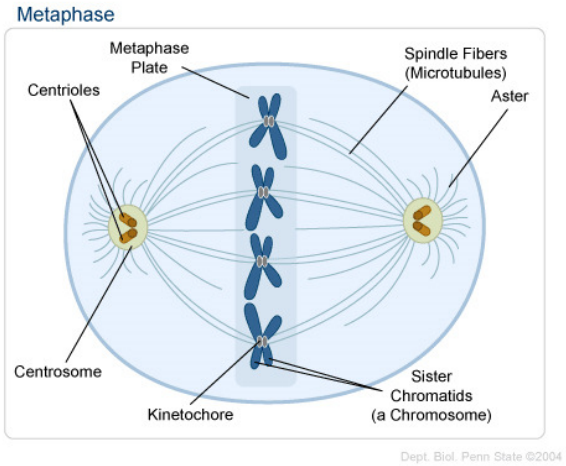


Figure 7: Diagram representing the structure of the mitotic spindle in metaphase.
The mitotic spindle in composed by two halves made mainly of microtubules, which divide by the chromosomes aligned in the equidistant line from both poles (metaphase plate). Microtubules binds chromosomes through a protein platform called kinetochores. At each pole there is a centrosome, the major nucleator of microtubules. From PennState, Eberly College of Science (https://online.science.psu.edu/biol110_sandbox_8862/node/8912).

1.7.1.Centromeres and kinetochores

The centromere is a special chromosomal locus that contains a specialized histone H3 variant called CenpA. Kinetochores are protein complexes assembled at centromeres, and function as attachment site to connect the chromosome to the microtubules in the mitotic spindle (Musacchio and Salmon, 2007). They are made of core structural elements, such as Ndc80–Hec1, microtubule binding proteins like dynein or the complex Ska and proteins that controls the mitotic checkpoint (also known as spindle assembly checkpoint, SAC) (Chan et al., 2012; Pfarr et al., 1990; Wigge and Kilmartin, 2001). SAC blocks anaphase onset until all kinetochores have correctly attached to spindle microtubules, preventing loss of chromosomes and aneuploidy. The signalling activity of the SAC depends on the formation of the mitotic checkpoint complex (MCC). This complex contains the SAC proteins Mad2, BubR1 and Bub3 bound to the SAC target Cdc20. A vast field

of research has been devoted to understanding the mechanisms behind the complexity of the interaction of SAC proteins with kinetochores (Bakhoum et al., 2009; Cleveland et al., 2003; Musacchio and Salmon, 2007; Sacristan and Kops, 2015).

1.7.2. Centrosomes

Centrosomes are microtubule-organising and nucleating centres in animal cells (Bettencourt-Dias et al., 2011). They influence the morphology of the microtubule cytoskeleton, especially during mitosis where the duplicated centrosomes mature into spindle poles (Hinchcliffe, 2014). Even though centrosomes play a central roles in assembling and orienting mitotic spindles, in many mammals and higher plants spindle do not have centrosomes (Schuh and Ellenberg, 2007). Therefore spindle assembly depends on others pathways (Schuh and Ellenberg, 2007) (see bellow).

Structurally centrosomes have a pair of cylindrical microtubule-based structures termed centrioles and, surrounded by the pericentriolar matrix (PCM). Each centriolar pair contains a mother and a daughter centriole, and their biogenesis is intimately correlated with the cell cycle (Figure 8).

Simultaneously with the DNA replication (S phase), each centriole functions as a template for the assembly of a new daughter centriole. The oldest centriole is known as the mother and is differentiated by having distal and subdistal appendages (Nigg and Raff, 2009; Nigg and Stearns, 2011) At onset of mitosis, centrosomes undergo maturation. During centrosome maturation, there is a dramatic increase in centrosome size and microtubule nucleation capacity, and enlargement of the PCM and acquisition of distal and subdistal appendages (G2/M phase). As a result of cell division, each daughter cell receives a single centrosome, reviewed in (Nigg and Stearns, 2011).

Importantly, mis-segregation can cause aberrant centrosome numbers, which has been linked with genome instability (Hardy and Zacharias, 2005).

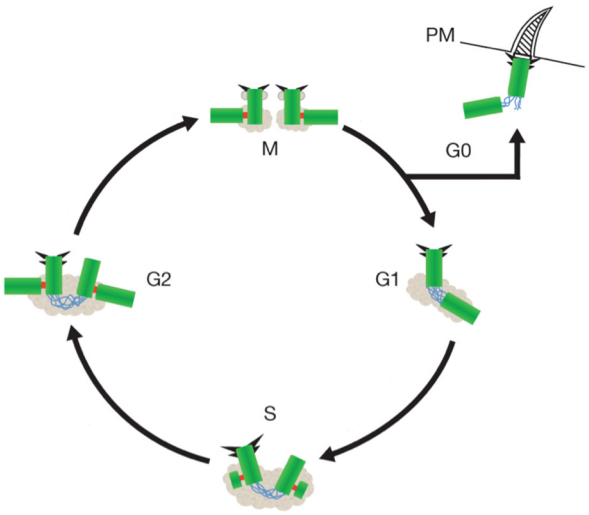


Figure 8: Centriole duplication cycle.
Schematic representation showing centrioles (green) and PCM (grey). Subdistal and distal appendages (triangles) are only presented in mother centrioles. Centrosomes are duplicated in S phase and reach full maturation in mitosis (M phase). In quiescent cells, G0, centriole associates with the plasma membrane (PM) and acts as a basal body to form a primary cilium. Adapt from (Nigg and Stearns, 2011).

Several proteins have been identified as critical in the control of centrosome number, biogenesis and its cycle. Recent work has shown that the human centrosomal protein, Cep192, is required for both centrosome maturation and spindle assembly (Gomez-Ferreria et al., 2007; Gomez-Ferreria and Sharp, 2008; Joukov et al., 2014). Cep192 works as a dominant mitotic PCM scaffolding, where it binds to Aurora A and to Plk1, two important kinases for centrosome maturation (Gomez-Ferreria et al., 2007; Joukov et al., 2014). Cep192 also forms a platform to assemble and activate complexes involved in microtubule nucleation, however it is not completely required as in its absence, microtubules are still able to assemble (Gomez-Ferreria et al., 2007).

1.7.2.1.Cilia

In non-cycling cells, centrioles can function as basal bodies in the formation of primary cilia and flagella (Bettencourt-Dias et al., 2011; Nigg and Raff, 2009). In interphase, the centrosome migrates and docks to the cell surface, where the mother-centriole can be modified into a basal body. From there, the cilia start to grow (Figure 8). Cilia are mechanical or chemical sensors from where signals are converted into transduction cascades regulating cell-polarity, differentiation, mobility and cell growth from development to tissues homeostasis (Satir and Christensen, 2007). Due to a wide variety of ciliary functions, defects in ciliar structure or proteins that are responsible

for cilia formation, maintenance or function result in a multiplicity of diseases and syndromes termed ciliopathies (Bettencourt-Dias, 2011; Bettencourt-Dias, 2007; Gerdes, 2009). Abnormal biogenesis of cilia can also result in cancer or developmental problems (Gerdes et al., 2009; Pazour and Witman, 2003). In addition, a number of proteins closely associated with ciliogenesis and cilia function are also implicated in cell cycle control. For example, Aurora A is not only a major kinase in the regulation of mitosis, but also one of best-studied disassembly mechanism of cilia. Aurora A kinase, primarily activated by the scaffolding protein HEF1/NEDD9, activates the tubulin desacetylase HDAC6 which destabilise the central microtubule structure of the cilium (axonema) and thus initiating cilia resorption (Pugacheva et al., 2007).

1.7.3.Microtubules

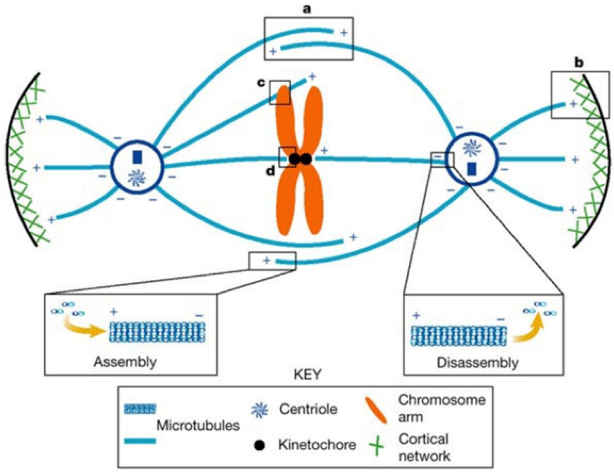


Figure 9: Microtubules classes within the mitotic spindle.
Spindle microtubules are mainly nucleated from the centrosomes, where all microtubules are orientated with their fast growing (plus) ends distal to the spindle poles. Interpolar microtubules (a) bundles exert forces capable of moving opposite spindle poles relative to one another. Astral microtubules (b) link spindle poles to the cell cortex and contribute to the positioning of the spindle relative to the cell cortex. K-fibres (d) move chromosomes relative to spindle poles. It is also possible to observe some microtubules (c) link to chromosome arms. From (Sharp et al., 2000)

Microtubules are complex polymers made of α/β-tubulin heterodimers. These heterodimers polymerise head to tail to form protofilaments, and 13 protofilaments assemble into a polar cylindrical structure. They are very dynamic, alternating between fast growth at the plus-ends (polymerisation), pausing, and rapid shrinkage (depolymerisation) (Sharp et al., 2000). Conversion from growth to shrinkage is defined as catastrophe, whereas the opposite switch is considered a rescue event. Their dynamics and polarity are dependent on GTP. Fast growing microtubule plus ends contain caps of GTP tubulin, but β-tubulin within the lattice will rapidly

hydrolyse GTP. Plus ends are fast growing, while the minus ends are less dynamic (Conde and Caceres, 2009). In addition to the intrinsic GTPase activity, nucleation and dynamics can be modulated by the microtubules associated proteins (MAPs) and motor proteins (Glotzer, 2009).

Throughout mitosis, microtubules are organised into distinct structures: k-fibres, astral and interpolar (or antiparallel) microtubules. Astral microtubules connect the spindle to the cortical membrane and contribute to the separation of spindle poles and positioning of the spindle relatively to the cell cortex. Cytokinesis is largely dependent on the central spindle, which is composed of an array of antiparallel microtubules that are bundled at their overlapping plus ends (Glotzer, 2009). The microtubule bundles that power chromosome movement and segregation are named k-fibres. They are force-generating units of 20-40 microtubules that run from the kinetochore to the spindle pole. Electron micrographs revealed that electron-dense inter-microtubules bridges are connected to these bundles by a network of microtubule connectors termed 'the mesh'. These bridges are hypothesised to allow the uniform transduction of forces throughout the microtubule bundle and to contribute to k-fibre stability (Nixon et al., 2015).

K-fibres formation are favoured by selective stabilisation of kinetochores-bound microtubules through the interaction with HURP, or MCRS-1 proteins (Meunier and Vernos, 2011; Sillje et al., 2006). HURP is a Ran-importin-regulated and Aurora A-dependent protein that localises at the vicinity of chromosomes (Sillje et al., 2006; Wong et al., 2008). This protein participates in interkinetochore tension and promotes chromosome congression by modulating the kinesin 18 (Wong et al., 2008; Ye et al., 2011).

Additionally, it was recently described that the shortest type of inter-microtubules bridges contain a ternary complex composed by Tacc3/ch-Tog/clathrin proteins, which cross-link adjacent microtubules and participate in the maintenance of these k-fibre structure and their stability (Booth et al., 2011; Nixon et al., 2015). K-fibre depolymerisation and destabilisation can be stimulated by the microtubule-depolymerising kinesins, Kif2b and MCAK (Bakhoum et al., 2009). This depolymerisation is antagonised by the MAP Tpx2. Tpx2 is required for microtubule

nucleation near kinetochores; an activity that also depends on Ran (Gruss et al., 2001; Kufer et al., 2002).

1.8.Spindle assembly

During mitotic spindle assembly, microtubules nucleated from the centrosomes capture kinetochores by making lateral connections where the kinetochore associates with the lattice of the microtubule rather than the plus-end (Hayden et al., 1990). As kinetochores are captured by additional microtubule plus-ends, stable microtubules bundles are generated, the k-fibres (McEwen et al., 1997). Selective stabilisation of these microtubules form a fusiform spindle in which the poles are focused at the centrosomes. Although centrosomes are a major microtubule-organising centre, they can also be nucleated and organised in the vicinity of chromosomes, where GTPase Ran has a key role (Bastiaens et al., 2006).

As described above, the nucleo-cytoplasmic transport directionality depends on the GTPase Ran (Hoelz et al., 2011). Ran can cycle into two distinct conformations, depending on the nucleotide binding (GTP- and GDP-bound forms). Its cycle is controlled by a GTPase-activating protein RanGAP, and by Ran's guanine nucleotide exchange factor (GEF), regulator of chromatin condensation 1 (RCC1). RCC1 binds directly to dsDNA and increases the rate of GTP-for-GDP exchange on Ran; RanGAP is found on the cytoplasmic side of the nuclear pore and accelerates GTP hydrolysis. Thus, due to the distinct localisations of the GEF and GAP, RanGTP is formed at a higher rate around chromosomes than at the cell periphery, where GTP hydrolysis occurs. Consequently, a diffusion-limited gradient of RanGTP is formed, Figure 10.

Similarly to nucleo-cytoplasmic transport, importin β can bind to proteins involved in spindle formation (SAFs); binding of RanGTP to importin induces the release of these factors and allows them to be active more close to the high chromosomal levels of RanGTP (Figure 10) (Bastiaens et al., 2006; Kalab and Heald, 2008). Numerous SAFs, such as Tpx2 (Gruss et al., 2001) MCRS1 (Meunier and Vernos, 2011) and NuMA (Nachury et al., 2001) are known to be RanGTP-regulated, reviewed in (Kalab and Heald, 2008).

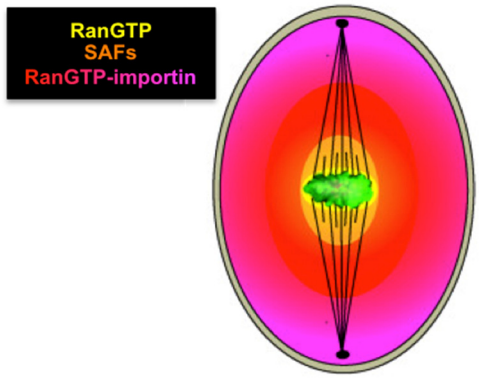


Figure 10: RanGTP gradient during mitosis.
Local production of RanGTP by chromatin bound RCC1 (yellow) that releases spindle assembly factors (SAFs) from importin β (red/pink). These generate a gradient important for the formation of spindle. Adapted from (Fuller, 2010).

1.8.1. Spindle assembly factors (SAFs)

Spindle assembly factors (SAFs), as the name suggests, assist in the assembly of the bipolar spindle either by directly interacting on microtubules, changing their dynamics, or by modifying the spindle lattice through cross-linking (Wittmann et al., 2001). SAFs can be microtubule associated proteins (MAPs) and/or motor proteins. In the Table 1 an overall description of the main functions of major SAFs within the spindle is presented. These proteins are mainly regulated by mitotic kinases (see below) (Glotzer, 2009) and/or a gradient of Ran (as described on the previous section) (Kalab and Heald, 2008).

Table 1: Overall description of the major SAFs acting in mitosis.

SAF	Mitotic function	Localisation at metaphase	References
Augmin complex	Centrosome and spindle integrity and microtubule bundling	Spindle microtubules	(Lawo et al., 2009) (Kamasaki et al., 2013) (Petry et al., 2013)
chTog	K-fibre stabilisation	Spindle microtubules, centrosomes and inter-microtubules bridges	(Booth et al., 2011) (Nixon et al., 2015) (Fielding et al., 2010)
Clathrin	K-fibre stabilisation	Cytoplasm and spindle microtubules	(Royle et al., 2005) (Booth et al., 2011) (Hood et al., 2013) (Lin et al., 2010)
Dynein-dynactin	MAP, pole focusing	Cytoplasm and spindle microtubules	(Merdes et al., 2000) (Silk et al., 2009)

Eg5/Kif11	Kinesin, antiparallel microtubule cross-linking, spindle integrity and centrosome separation	Spindle microtubules	(Ma et al., 2011) (Giet and Prigent, 2000) (Heck et al., 1993)
HURP	MAP, k-fibre stabilisation, chromosome congression and interkinetochore tension	K-fibres	(Sillje et al., 2006) (Wong et al., 2008)
Kif15	Kinesin, spindle elongation and integrity and centrosome separation	Spindle microtubules	(Tanenbaum et al., 2009)
Kif18	Kinesin, chromosome congression, k-fibres dynamics	K-fibres	(Ye et al., 2011) (Mayr et al., 2007)
Kif2a	Kinesin, microtubule dynamics	Spindle microtubules	(Jang et al., 2009) (Ganem and Compton, 2004)
Kif2b	Kinesin, microtubule dynamics	Spindle microtubules	(Bakhoum et al., 2009)
MCAK/Kif2c	Kinesin, microtubule dynamics	Kinetochores and spindle microtubules	(Domnitz et al., 2012) (Wordeman et al., 1999)
MCRS1	K-fibre stabilisation	K-fibres	(Meunier and Vernos, 2011) (Petry and Vale, 2011)
NuMA	MAP, pole focusing	Spindle poles	(Silk et al., 2009) (Merdes et al., 2000)
Nup188	K-fibre stabilisation	Spindle microtubules	(Itoh et al., 2013)
Nup98	Microtubules dynamics	Spindle microtubules	(Cross and Powers, 2011)
Tacc3	K-fibre stabilisation and tension, microtubule dynamics, centrosome clustering	Spindle microtubules, centrosomes and inter-microtubules bridges	(Booth et al., 2011) (Nixon et al., 2015) (Fielding et al., 2010) (Burgess et al., 2015)
Tpx2	MAP, microtubule bundling and spindle integrity	Spindle microtubules	(Wittmann et al., 2000) (Petry et al., 2013)

1.9.Forces within the spindle

Active forces within the spindle are involved in chromosome congression and segregation (Dumont and Mitchison, 2009). Polymerisation dynamics of microtubules convert chemical energy into mechanical work. Hence, microtubule fibres can push by polymerising and pull by depolymerising. Also, forces can be generated from spindle component properties, such as elasticity and molecular friction forces (Dumont and Mitchison, 2009). In this context, the movement of the chromosomes at anaphase has been interpreted as a consequence of the microtubules pulling the kinetochores towards the spindle poles like strings. Also, kinetochore and cohesin can be viewed as a set of interacting springs (elastic properties, Figure 11) (Vladimirou et al., 2011). Tension forces on kinetochores become evident when k-fibres are depolymerised and the distances between sister kinetochores decrease (recoil of a spring) (Waters et al., 1996). Additionally, pulling forces from the cell cortex through astral microtubules may also play a role in kinetochore tension (Dumont and Mitchison, 2009).

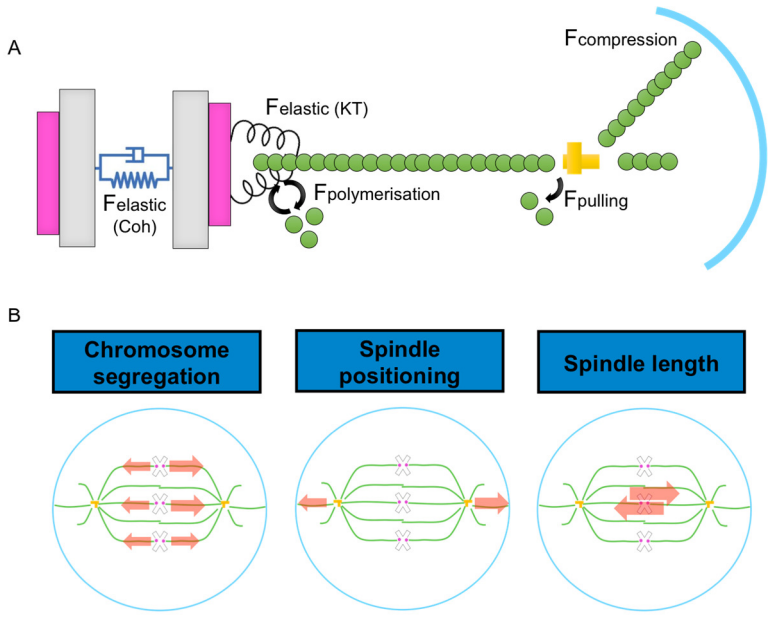


Figure 11: Forces within the mitotic spindle.
(A) Schematic diagram representing the forces within the mitotic spindle. Chromosomes are segregated by pulling forces, and tension on kinetochores is generated by a combination of compression and polymerisation forces. Sister chromatids (light grey) are held by cohesin (Coh, blue) and connected to microtubules (green) through kinetochores (KT, pink and black coils). Both Coh and KT have elastic properties and the distance between the sister chromatids can reflect the balance between tension and compression forces within the spindle. (B) Spindle forces are important for regulating spindle positioning and length and chromosome segregation. Adapt from (Dumont and Mitchison, 2009) and (Civelekoglu-Scholey and Cimini, 2014).

In the majority of systems, while k-fibres are mainly straight, non-kinetochore-microtubules are curved, which may be caused by compression forces. According with this model, if k-fibres are ablated, the spindle length will increased as a result of straitening of the interpolar microtubules. DeLuca and collaborators proved this hypothesis after genetic ablation of k-fibres (DeLuca et al., 2006), however results are less consistent if only a few k-fibres are ablated by UV microbeam (Zheng, 2010).

These observations show that microtubule dynamics cannot only generate forces, but also be affected by them, making it very difficult to understand the interplay between force production/response and assembly dynamics (Figure 11). By balancing forces within the spindle, cells with identical size assemble spindles with similar length. Similarly, the spindle position within the cell is dependent upon the balance of forces (see bellow) (McNally, 2013).

1.9.1. Spindle matrix

There have been many observations over the years that non-microtubule components can influence the stabilisation of the spindle and its mechanical properties. Mechanical perturbation experiments suggest that spindle microtubules are under external compression as discussed above (Dumont and Mitchison, 2009; Poirier et al., 2010; Zheng, 2010). Also, the presence of this matrix was suggested from unbalanced k-fibres ablation experiments (Pickett-Heaps et al., 1997). After several k-fibres were ablated by UV microbeam in one half of the spindle, the compression forces from the spindle matrix caused a shortening of the irradiated half of spindle (Pickett-Heaps et al., 1997). The unbalanced number of k-fibres on each side of the spindle during metaphase causes the poleward movement of chromosomes and progression into anaphase (see Figure 12B) (Poirier et al., 2010). These results could be justified by the presence of an elastic spindle matrix (Lydersen and Pettijohn, 1980; Schweizer et al., 2014). The complexity of the spindle matrix has made the study of its molecular and structural composition very difficult, as well as its function, which contributes to the debate of its role and even existence (Schweizer et al., 2014; Zheng, 2010). Nonetheless, according to the current model, this matrix helps to stabilise spindle microtubules and to concentrate and/or activate spindle assembly regulators (Schweizer et al., 2014; Zheng, 2010).

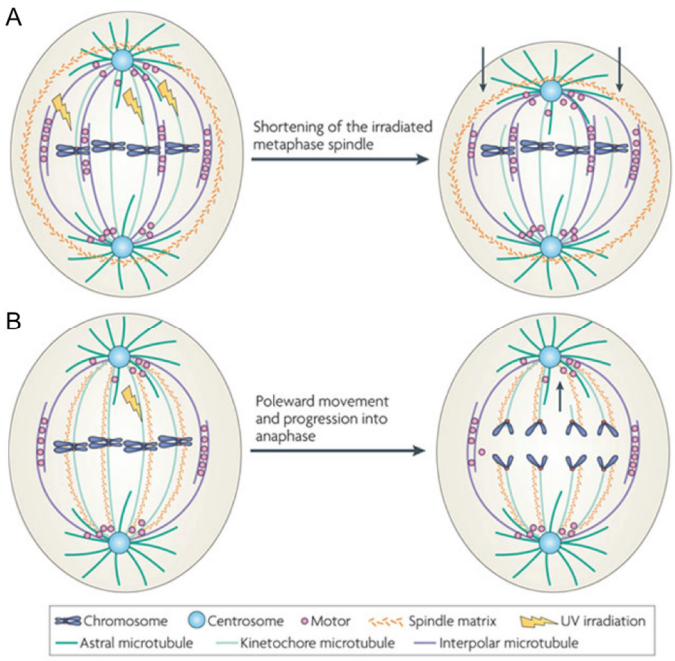


Figure 12: Elastic spindle matrix.
An elastic spindle matrix surrounds spindle microtubules (A) along k-fibres (B). In A several, but few, k-fibres where cut by ultraviolet (UV) irradiation near to the metaphase plate. After irradiating, the uncut microtubules in this half curve back, causing shortening of the half spindle. In B, UV irradiation of the indicated k-fibres did not block their correspondent kinetochore from moving to spindle poles during anaphase (represented by the arrow). Both experiments suggest that the elastic spindle matrix is normally stretched by spindle microtubules. When this stretching force is reduced, the spindle matrix would collapse onto spindle microtubules, causing them to bend and buckle. From (Zheng, 2010).

The spindle matrix is defined as a non-microtubule scaffold lattice that surrounds the microtubule spindle apparatus during mitosis (Schweizer et al., 2014). NuMA was the first protein to be described in both the interphase nucleus and at mitotic spindle poles (Lydersen and Pettijohn, 1980). Ten years later NuMA was the first protein to be considered as part of a putative lattice called the "spindle pole matrix" (Dionne et al., 1999). As described above it is now known to be a SAF that is responsible for focusing of spindle by tethering microtubule minus ends (Merdes et al., 2000). Another well-studied spindle matrix component is the Megator (Mtor) protein found in *Drosophila melanogaster* (a homolog of the human NUP Tpr) (Lince-Faria et al., 2009). So far, only a subset of proteins have been associated with the spindle matrix, namely NuMA, NUPs (such as Megator, chromator), lamins and vesicles from Golgi and ER (Ding et al., 2009; Lince-Faria et al., 2009; Ma et al., 2009; Scholey et al., 2001; Zeng et al., 1994). From proteomic analysis of the *Xenopus* spindle matrix, Zeng and collaborators have identified new components of this matrix, such as BuGZ, which facilitates spindle microtubule assembly and metaphase-to-

anaphase transition (Jiang et al., 2015a; Jiang et al., 2014; Jiang et al., 2015b). Recently it was published that this nuclear-derived proteinaceous matrix function as an organelle-exclusion envelope that assists mitosis, suggesting an additional role of this matrix during mitosis (Schweizer et al., 2015).

1.10.Regulation of mitosis by phosphorylation

During each cell cycle, faithful chromosome segregation is tightly maintained by numerous regulatory proteins, most notably kinases, which regulate the phosphorylation status of numerous proteins in a cell-cycle-dependent manner. Eukaryotic cells use post-translational modifications (PTMs) as an essential mechanism to diversify their protein functions and dynamically coordinate several signalling networks, such as entering or exiting mitosis. One of the most studied modification is protein phosphorylation, where an addition of a phosphate group to an amino acid residue, in particular tyrosine, serine and threonine, takes place. This addition is carried out by kinases and counteracted by phosphatases (Nigg, 2001). To date, a relatively small number of conserved serine/threonine protein kinase families involved in mitosis were identified: cyclin-dependent kinase (Cdk), Polo, Aurora and NIMA-related kinase families (Neks), reviewed in (Bayliss et al., 2012).

1.10.1.NIMA-related kinases (Neks)

In the middle 70s, Morris and collaborators identified a gene in the filamentous fungus *Aspergillus nidulans* that when mutated cells failed to enter mitosis. This gene encodes the never-in-mitosis A (NIMA) protein, a serine/threonine kinase that is required for mitotic entry (Oakley and Morris, 1983). Since then, NIMA-related kinases, or NEKs, have been identified in most eukaryotes. So far, eleven members of this family (NEK1 to NEK11) have been identified. Although there is no evidence that human NEKs are critical for mitotic entry, they have been implicated in several aspects of mitotic progression (Moniz et al., 2011). For instance, NEK2, NEK6, NEK7 and NEK9 participate in the assembly of the bipolar spindle and centrosome separation/splinting (Bertran et al., 2011; Faragher and Fry, 2003; O'Regan and Fry, 2009). NEKs also have roles in chromatin condensation (Faragher and Fry, 2003), nuclear envelope breakdown, SAC signalling and cytokinesis (Moniz et al., 2011; O'Regan and Fry, 2009).

1.10.2.Cyclin-dependent kinases (Cdks)

Cyclin-dependent kinases (Cdks) represent one of the major well-studied kinase families involved in the cell cycle. As the name suggests, these kinases are completely inactive without a cyclin

partner protein. After binding of a cyclin, Cdk is phosphorylated by a Cdk-activating kinase, to become active. Cdk protein levels do not change throughout the entire cell cycle. On the contrary, cyclin protein levels "cycle" during the cell cycle, being expressed in specific stages. Recent work has revealed some overlap and essential functions between the members of the Cdk family of proteins, adding an extra layer of complexity in the regulation of cell division, reviewed in (Hochegger et al., 2008).

1.10.3.Polo-like kinases (Plks)

To date, there are four polo-like kinases (Plks, Plk1-4) described with known functions in several stages of mitosis and meiosis (Barr et al., 2004). Structurally, they have a similar architecture, a serine/threonine kinase domain at the N-terminus and a polo box signature motifs at the C-terminus (with distinct localisations between the kinases) (Bayliss et al., 2012; Glover, 2005). In the case of Plk1, its localisation is dynamic within the cell cycle. It localises at centrosomes during G2, then at kinetochores and spindle poles during prometaphase and metaphase, and finally at the central spindle during anaphase–telophase. Its localisation is associated with distinct role in several processes, including mitotic entry, centrosome maturation, microtubule nucleation, bipolar spindle assembly and cytokinesis, reviewed in (Barr et al., 2004).

1.10.4.SAC related kinases: Mps1, Haspin and Bub proteins

The signalling activity of the SAC depends on the formation of the mitotic checkpoint complex (MCC). Monopolar spindle-1 (Mps1) kinase family are not only known by a conserved role in spindle checkpoint (SAC) signalling, but also for participating in proper chromosome alignment at the metaphase plate, and for ensuring the fidelity of chromosome segregation (Liu and Winey, 2012). Mps1 mediates the recruitment of MCC components, including Mad1, Mad2, Bub1, BubR1 and Bub3, reviewed in (Bayliss et al., 2012). Nonetheless, there are two known members of the MCC complex with serine/threonine kinase activity: Bub1 and Bub1B/BubR1 (Bolanos-Garcia and Blundell, 2011). The precise role of their catalytic activities towards SAC is still under investigation.

Finally, Haploid germ-cell-specific nuclear protein kinase (Haspin) is a newly discovered serine/threonine protein kinase that when depleted results in defective chromosome alignment at the metaphase plate and a delay in mitotic exit due to activation of the SAC (Higgins, 2010).

1.10.5. Aurora kinases

Glover and collaborators identified the first Aurora kinase (Aurora A) in a genetic screen performed in *Drosophila* for genes required to maintain centrosome and spindle assembly functions (Glover et al., 1995). After twenty years, much more is known about this family of kinases, in particular that there are three members in mammals (Aurora A, B and C), two in *Drosophila,* and one in fungi, which is more related to the Aurora B kinases in higher organisms (Vader and Lens, 2008).

Aurora kinases contain D-box and KEN regulatory motifs at their N- and C-terminus. At the central kinase domain there are key regulatory motifs for the catalytic activity. In their central kinase domains there are regulatory motifs key for activity. These kinases are activated by T-loop autophosphorylation (Figure 13). Several cofactors have been identified as essential for proper activation and function of all three Aurora kinases (Goldenson and Crispino, 2015).

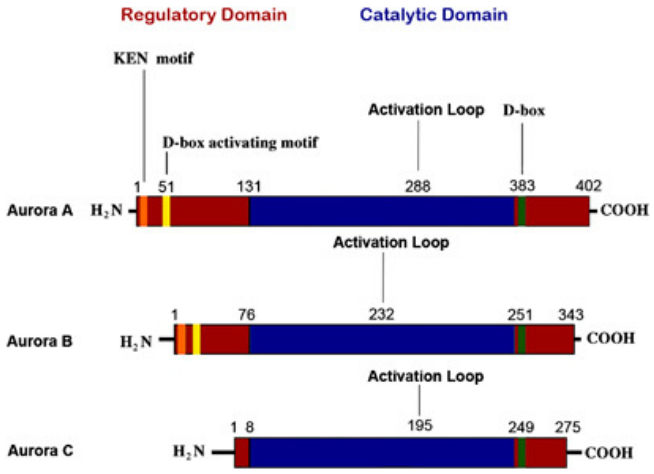


Figure 13: Structure and domains of the Aurora kinase family.
Both N- and C-terminus domains contain D-box and KEN regulatory motifs while the central kinase domain have key regulatory motifs for the catalytic activity such as the activation (T-loop) residue. From (Goldenson and Crispino, 2015).

Although all three Aurora kinases are involved in cell division, Aurora A, but not B or C, regulates bipolar spindle assembly, centrosome maturation and separation. Aurora B regulates chromosome condensation, SAC and chromosomes attachments, and cytokinesis (Adams et al., 2000; Vader

and Lens, 2008). Recently it was shown that Aurora B has a direct role in establishing the SAC checkpoint together with Mps1 and Hec1, a protein of the kinetochore phosphorylated by Aurora B (Saurin et al., 2011).

As far as Aurora C is concerned, it is still very poorly understood. It is not ubiquitously expressed, and it was proposed to complement Aurora B kinase function in mitotic cells. It is also known to participate in cytokinesis and mouse spermatogenesis (Kimmins et al., 2007; Sasai et al., 2004).

1.10.5.1. Aurora A

Aurora A function and expression peaks during early mitosis. Aurora A kinase activity is dependent on the phosphorylation of the threonine 288 (T288) within the activation T-loop, Figure 13 (Littlepage et al., 2002). The most studied and best-characterised cofactor of Aurora A is Tpx2. Tpx2 was initially characterised as a SAF (Table 1) (Petry et al., 2013; Wittmann et al., 2000). Later on, it was shown to bind to Aurora A, modulating Aurora A localisation and activity within the spindle (Kufer et al., 2002). After NEBD, active RanGTP (Trieselmann et al., 2003; Tsai et al., 2003) releases Tpx2 from the importin-β. Once free, Tpx2 binds to Aurora A, which induces a conformational changes in Aurora A modifying the position of T288 stimulating autophoshorylation (Bayliss et al., 2003; Satinover et al., 2004; Zorba et al., 2014). Aurora A dynamically associates with the centrosome and spindle poles (Lioutas and Vernos, 2014), however TPX2 is not involved in the targeting of Aurora A to the centrosome (Kufer et al., 2002). Several studies have shown that Aurora A targeting and activation within the spindle and centrosomes are very promiscuous and dependent on a large number of proteins, such as the focal adhesion-associated signaling protein (HEF1) (Bayliss et al., 2012; Pugacheva and Golemis, 2005). HEF1 during mitosis relocalises into to spindle, where it was shown to be associated with the control of Aurora A activation (Pugacheva and Golemis, 2005). Other studies have described that Ajuba, a scaffolding protein, is also an interactor and activator of Aurora A (Hirota et al., 2003). It was shown that its depletion in *Drosophila* did not perturb Aurora A activity, but affected its recruitment to the centrosome (Sabino et al., 2011).

Nucleophosmin/B23, Bora and Pak1 are others upstream activator of Aurora A kinase (Reboutier et al., 2012)(Bruinsma et al., 2014)(Zhao et al., 2005). Pak1 is a member of the P21-activated kinases (Paks), a family of serine/threonine kinases with functions in mitosis (Maroto et al., 2008). Pak1 promotes phosphorylation of Aurora A (Zhao et al., 2005) and Plk1(Maroto et al., 2008). On the other hand, Plk1 was shown to be required for Aurora A association with centrosomes (Bruinsma et al., 2014; Joukov et al., 2010; Joukov et al., 2014).

Centrosomal Aurora A localisation is dependent on CEP192 (Joukov et al., 2010; Joukov et al., 2014). This centrosomal protein directly interacts with Aurora A, and allows the formation of homodimers and homo-oligomers of Aurora A (Joukov et al., 2010). Recently, CEP192 was shown to bind to Plk1 prior to Aurora A activation (Joukov et al., 2014). Further studies are necessary to better clarify the Aurora A-Plk1 signalling cascade; nonetheless, CEP192 was suggested to be a major activator of Aurora A at the centrosome via Plk1 (Joukov et al., 2010; Joukov et al., 2014).

Aurora A dynamically associates with the centrosome and spindle poles (Lioutas and Vernos, 2014). There, it participates in centrosome separation and maturation, mitotic entry, and spindle assembly and integrity, namely by promoting microtubule nucleation and stabilisation (Carmena et al., 2009; Dutertre et al., 2002; Lioutas and Vernos, 2014). Aurora A's role in mitosis is exerted by regulating the phosphorylation levels of numerous SAFs (Sardon et al., 2010). Aurora A phosphorylates and recruits MAPs such as the kinesin Eg5 and the acidic coiled-coil protein, Tacc3 to the centrosome and spindle (Cheeseman et al., 2011; Giet et al., 2002; Giet and Prigent, 2000; Lioutas and Vernos, 2013). Similarly, Aurora A interacts and phosphorylates the gammaTURC adaptor protein NEDD1 (Pinyol et al., 2013). The Augmin complex Hice1 protein is also phosphorylated by this kinase to inhibit microtubule nucleation (Pinyol et al., 2013; Tsai et al., 2011). Aurora A regulates the activity of HURP at the k-fibres stabilisation by controlling the accessibility of its microtubule-binding domain through phosphorylation (Wong et al., 2008). Proteomics studies uncovered new potential Aurora A substrates including NuMA during spindle assemble (Kettenbach et al., 2011; Sardon et al., 2010). These new proposed substrates suggested its participation in cytokinesis, intra cellular transport, etc (Sardon et al., 2010).

Full Aurora A inhibition by specific kinase inhibitors or RNA-interference-mediated silencing produces mitotic spindle pole fragmentation, microtubule hyperstabilisation and disruption of the microtubule flux, which is necessary for proper bipolar spindle assembly (Asteriti et al., 2011; Wang et al., 2008).

While the majority of studies focus on Aurora A's role are mainly studied in mitosis, this kinase also contributes in the regulation of non-mitotic functions. In non-cycling cells, Aurora A participates in polarity formation, microtubule organisation during interphase neurite elongation (Yamada et al., 2010) and ciliary resorption (Plotnikova et al., 2012; Pugacheva et al., 2007). Also, Aurora A overexpression or amplification is commonly found in tumours (Vader and Lens, 2008).

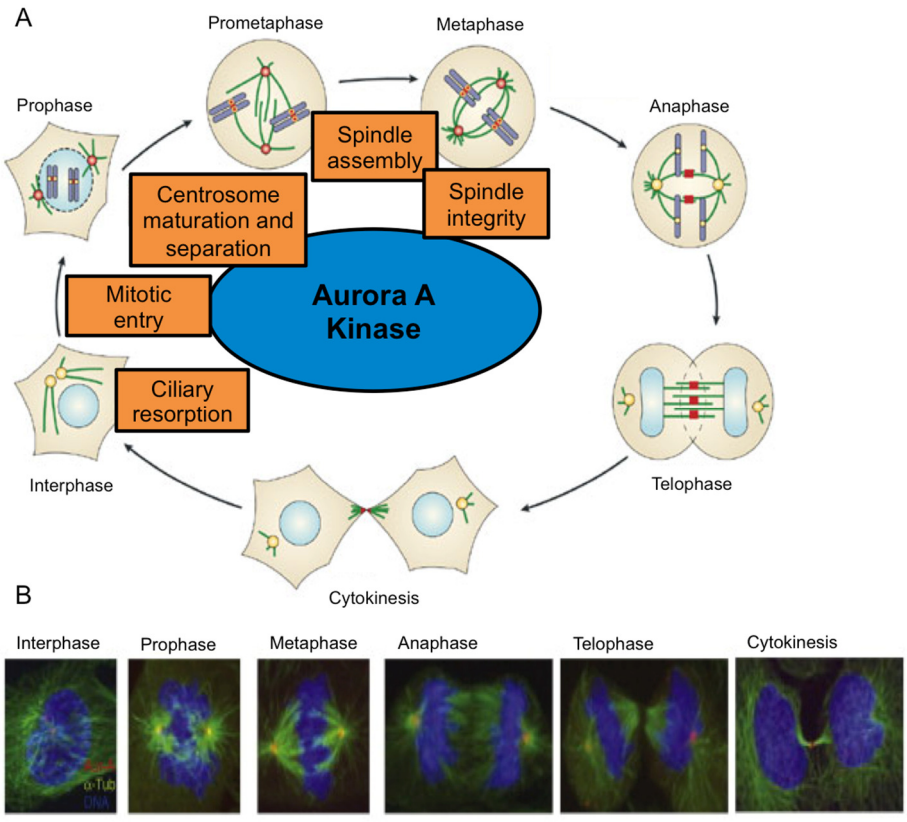


Figure 14: Functions of the Aurora A kinase.
(A) Schematic representation of the different phases of cell division and associated functions of Aurora A. (B) Localisation of Aurora A. Immunofluorescence images of mitotic cells, where tubulin is showed in green, DNA in blue and Aurora A in red. Adapted from (Vader and Lens, 2008) and (Barr et al., 2004).

1.11.Meiosis

The process of cell division in sexually reproducing organisms triggers a complex cellular programme that creates two highly specialised meiotic germ cells: the oocyte and the sperm. In contrast to mitotic cells, germ cells undergo meiosis in two consecutive rounds of cell division, meiosis I and II, without reduplication of the genome. Growth and maturation of both male and female gametes culminates in the production of fertilisation-competent eggs and sperm. Meiosis reduces chromosome content from diploid to haploid and allows for homologous recombination. Ultimately, a functional gamete is capable of undergoing fertilisation and giving rise to a developed embryo (Figure 15), reviewed in (Clift and Schuh, 2013; Ohkura, 2015). To become a fertilisable egg, an oocyte has to eliminate half of the chromosome content into a small polar body. The other half will come from the sperm during the fertilisation process.

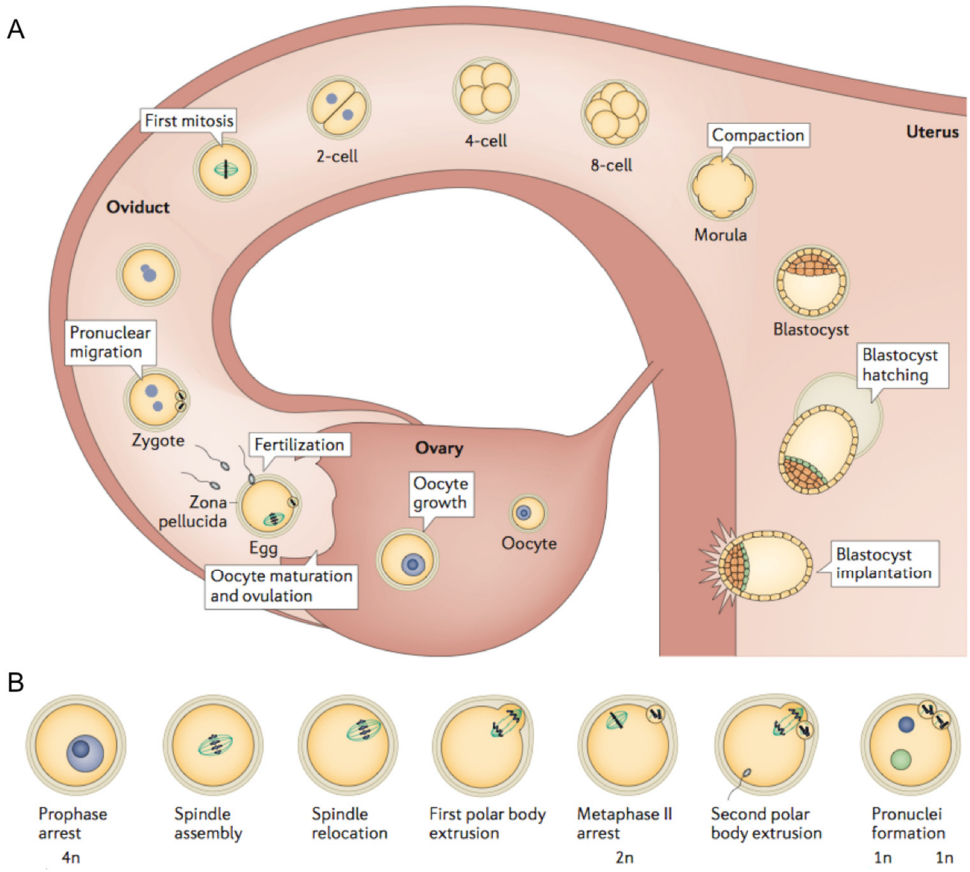


Figure 15: From oocyte to embryo.
(A) **Diagram representing an overview from oocyte to embryo. Eggs are fertilised at the oviduct to form the zygote. After several divisions, the dividing cells of the embryo assume a spherical and compact shape called a morula. Then, a cavity is formed and two distinct populations are visible on the embryo. At this stage the embryo is termed blastocyst, which it migrates to its implantation. (B)**

Maturation of mammalian oocyte. A prophase I arrested oocyte, with a 4n begins to maturate. Following germinal vesicle breakdown (GVBD), meiosis I spindle assembles. Then it relocates to the oocyte surface where it extrudes the first polar body. Immediately after meiosis I anaphase, a second spindle assembles around the remaining chromosomes (2n). The now matured egg arrests in metaphase II awaits for fertilisation. Upon fertilisation, meiosis is reinitiated (meiosis II) and a second polar body is extruded leaving a haploid female pronucleus (1n). From (Clift and Schuh, 2013).

1.11.1.Oocyte maturation

Oocyte maturation is the process by which an ovarian germ cell becomes an egg, and it encompasses a number of critical events. At the onset of meiosis I, stored oocytes are arrested in meiotic prophase I in primordial follicles. Follicles are functional compartments for the growth of the oocyte. In a subsequent stage, under the action of follicle stimulating hormone (FSH), follicles increase in size. Under the influence of gonadotropins, once every menstrual cycle, fully grown oocytes mature into fertilisable eggs. This process can be stimulated with exogenous gonadotrophins and partially supported by *in vitro* culture. Importantly, the majority of the studies in the field are performed in mouse, and a major challenge in the future will be to understand whether human oocytes employ similar mechanism of mouse oocyte maturation (Clift and Schuh, 2013; Li and Albertini, 2013).

1.11.1.1.Acentrosomal spindle assembly in mouse oocytes

After long periods in prophase I arrest, meiotic resumption is initiated in the follicle by the breakdown of the nuclear envelope of the germinal vesicle (GV) triggered by the Maturation Promoting Factor (MPF) (Howe and FitzHarris, 2013). Along with GVBD, a microtubule spindle assembles around the chromosomes. The spindle is assembled in the absence of centrosomes, as female meiotic cells do not contain canonical centriole-containing centrosomes (Verlhac et al., 2000).

Shortly after meiotic maturation resumes, numerous acentriolar MTOCs (aMTOCs), with microtubule nucleation properties similar to centrosomes, are formed *de novo* from a cytoplasmic microtubule network (Schuh and Ellenberg, 2007). These aMTOC appear to contain gamma-tubulin and pericentrin (Schuh and Ellenberg, 2007) however it is not clear if their formation is dependent or not on Ran (Dumont et al., 2007b; Schuh and Ellenberg, 2007). Microtubules are predominantly nucleated from aMTOC, but their numbers increase in a Ran-dependent manner

after GVBD (Li and Albertini, 2013). As in mitosis, Ran mediates important features of the spindle assembly, namely the spindle length and timing of bipolarity (Dumont et al., 2007b).

1.11.1.2. Asymmetric spindle positioning in mouse oocytes

In the large majority of animal cells the spindle is located at the centre of the cell, and after division two daughter cells of equal size are generated; asymmetrical spindle positioning generates polarised cells. Mouse oocytes have extremely asymmetrical spindle positioning (Brunet and Maro, 2007; Fabritius et al., 2011). During meiosis I, after assembly of the spindle in the centre, the spindle migrates towards the nearest oocyte cortex. Once one of the spindle poles reaches the cortex, the spindle rotates and adopts a perpendicular orientation relative to the cortex (Fabritius et al., 2011; McNally, 2013). At anaphase onset the spindle is stably anchored to the cortex, and half of the chromosome mass is extruded into small non-developing polar bodies (Fabritius et al., 2011; McNally, 2013). This asymmetric division preserves most of the cytoplasmic content in the oocyte, which is essential for fertilisation (Chaigne et al., 2012; Fabritius et al., 2011).

Several recent studies in mouse oocytes showed that asymmetric spindle positioning is dependent upon actin and its nucleation factors Formin-2 (Azoury et al., 2008; Dumont et al., 2007a), Spire1 (Pfender et al., 2011) and Spire2 (Dumont et al., 2007a; Schuh and Ellenberg, 2008; Verlhac et al., 2000; Yi and Li, 2012). Further studies are required to better understand the mechanisms that govern spindle assembly and positioning during oocyte maturation.

After polar body extrusion, the spindle is reassembled into a bipolar structure and the oocyte remains in metaphase II arrest until fertilisation by a sperm. Infertility can be genetically caused by defective meiotic divisions (Ohkura, 2015).

1.11.2. Embryo

Following oocyte maturation, eggs are fertilised in the oviduct to form the zygote. After fertilisation, eggs arrested in metaphase II (2n) undergo sister chromatid segregation (1n). From there both male and female haploid pronuclei (1n+1n) are formed and migrate towards each other before the first mitotic division. After several mitotic divisions, smaller embryonic cells, termed

blastomeres, are produced. Later on, they form a compact structure called a morula. During subsequent divisions, two distinct populations of cells are generated, and the embryo matures into a blastocyst. At this stage, the blastocyst migrates into the uterus, where it implants into the uterine wall (Clift and Schuh, 2013).

1.12.Objectives

Several Nucleoporins (NUPs) have been shown to play additional functions within the cell beside their first described function in transport activities (Franks and Hetzer, 2013; Light and Brickner, 2013; Mendjan et al., 2006). It is now accepted that NUPs have roles during cell division (Chatel and Fahrenkrog, 2011; Gigliotti et al., 1998; VanGompel et al., 2015) and in cilia (Kee et al., 2012; Kee and Verhey, 2013). Furthermore, several NUPs have also been associated with different diseases (Cronshaw and Matunis, 2004), increasing the attention on them and their roles in the cell.

Triple A syndrome is a complex and multisystemic autosomal recessive disease associated with ALADIN (Tullio-Pelet et al., 2000). Much of what it is known about ALADIN function comes from studies in interphase, particularly on its role in oxidative stress response, RNA import and steroidogenesis in the context of triple A syndrome (Juhlen et al., 2015; Kind, 2010; Kind et al., 2009; Kiriyama et al., 2008).

The work presented in this thesis aimed to further expand the functional roles of ALADIN in the context of cell division.

Mitosis is a critical and highly orchestrated event in the cell cycle that involves hundreds of proteins. Multiple NUPs, which are part of the nuclear pore complex during interphase, are involved in mitosis, and play key roles in the regulation of this process (Chatel and Fahrenkrog, 2011). Here it was determined whether the NUP ALADIN was a new related NUP in mitosis (Chapter 3).

Additionally, currently understanding of how mutations of ALADIN cause the triple A syndrome is very limited (Cronshaw and Matunis, 2004; Huebner et al., 2000; Prasad et al., 2014; Sarathi and Shah, 2010). Hence, it was also sought to determine if ALADIN's role in mitosis could be related with triple A syndrome (Chapter 3).

Meiosis is a special cell division type that generates gametes, and it is necessary for reproduction. Meiosis shares many mechanisms and regulators with mitosis (Ohkura, 2015). Therefore, it was

also addressed whether ALADIN could play a role in mammalian meiosis, using mouse oocyte as a model (Chapter 4).

Cilia assembly has a close relationship with the timing of cell division (Kobayashi and Dynlacht, 2011). Recently several NUPs have been shown to localise at the cilia (Kee et al., 2012; Kee and Verhey, 2013). Therefore, initial studies were performed to evaluate whether ALADIN could be another NUP with a role in cilia function. Finally, it was addressed if ALADIN could be related with ciliary functions in the context of triple A syndrome (Chapter 5).

2.Materials and Methods

2.1.Cell culture, siRNA, plasmid transfections and drug treatments

The fibroblast cell lines (p.S263P, p.Q387X and WT ALADIN) were previously described (Kind, 2010). The HeLa cell line stably expressing GFP-ALADIN was previously described (Kind et al., 2009), and the HeLa cell line stably expressing cell PA-GFP-α-tubulin and RFP-H2B was a gift from A. McAinsh (University of Warwick, Coventry, UK (Samora, 2011)). Both HeLa GFP-p150 and Aurora A-LAP were expressed from a BAC clone using the endogenous promoter, a gift from A. Hyman, (Max Planck Institute for Molecular Cell Biology and Genetics, Dresden, Germany) (Poser et al., 2008). All cell lines were grown in Opti-MEM + GlutaMAX supplemented with 10% fetal bovine serum and 100 U/mL penicillin, and 100 µg/mL streptomycin at 37° C with 5% CO_2 in a humidified incubator, with the exception of fibroblasts, where DMEM + GlutaMAX media was supplemented with 0.25 µg/mL Amphotericin B (all media and supplements were from Life Technologies).

To deplete ALADIN, $2.5x10^5$ cells were reverse transfected with 70 nM of Duplex Oligonucleotides 5'–UGAGUUUGCAAUUUCAUUUAGCACC–3' or 5'–AUCUCUUCCACACUUGCUCCCGGUG–3' (Stealth RNAi Life Technologies and Sigma) using Lipofectamine RNAiMAX (Life Technologies) for 48 h with a second RNAi forward transfection after 24 h. A low-GC Duplex siRNA (Life Technologies) was used as negative control. During transfection, cells were maintained in antibiotic-free complete culture media.

Transient transfections were performed using Fugene HD transfection reagent (Promega) according to the manufacturer's instructions. Typically, cells were transfected with a ratio of 3:1 transfection reagent:DNA for 48-72 h before assaying. GFP–ALADIN plasmid is described (Krumbholz et al., 2006) and pEGFP-C1 (Clontech) was used as empty GFP vector.

To increase the number of mitotic cells for immunostaining, cells were either incubated with 10 µM of MG132 (Calbiochem) for 4 h or with 2.5 ng/ml of RO-3306 (Calbiochem) overnight to arrest them at the G2/M border before releasing them into MG132 for two hours prior to fixation.

For live-imaging experiments or western-blot analysis, cells were treated with 2.5 ng/mL of RO-3306 or 5 μM S-Trityl-L-cysteine (STLC; Enzo-life-science) – or monastrol for 16-20 h. If metaphase arrest was required, cells were treated with MG132 for 2 h after the removal of RO-3306. For Aurora A inhibition, cells were treated with 1.5 ng/mL MLN8237 for 40 min (Selleckchem).

2.2.Mouse oocyte and sperm collection and culture

C57BL/6 background WT ($ALADIN^{+/+}$) and KO ($ALADIN^{-/-}$) were previously described (Huebner, 2006). Animals were bred and maintained under pathogen-free conditions at the Experimental Center of the Medizinisch-Theoretisches Zentrum of the Medical Faculty at the Dresden University of Technology according to approved animal welfare guidelines.

Oocytes were isolated from adult mice by puncturing the ovaries with needles in M2 medium supplemented with 1 μM milirinone to ensure prophase arrest. Surrounding follicle cells were removed by mouth pipetting. Resumption of oocyte maturation was induced by wash out the milirone. Then, for immunofluorescence analysis oocytes were incubated in M2 media covered by paraffin oil and maintained in a 5% CO_2 atmosphere at 37°C with humidity control until the desired stage (metaphase I: 6 h, metaphase II: 12 h).

For *in vitro* fertilisation (IVF) experiments, oocytes were also matured using super ovulation techniques. Briefly, female mice (21-27 days of age) kept in a 12 hour light-dark cycle were intraperitoneal treated 44 h before the extraction (day 0) with 5 IU pregnant mare serum gonadotropin (PMSG) and 12 h before day 0 with 5 IU human chorionic gonadotropin (HCG). At day 0, oocytes were released from the cumulus complex. Spermatozoa were extracted from adult male mice by dissecting the epididymis and tail tip.

2.3.Live cell imaging

All live cell imaging of HeLa cells was performed on a DeltaVision microscope (Applied Precision) outfitted with an environmental chamber to maintain 37°C, and cells were imaged in

Leibovitz's L-15 Medium supplemented 10% fetal bovine serum and 100 U/mL penicillin, and 100 µg/mL streptomycin.

For analysis of tubulin poleward flux, metaphase HeLa cells stably expressing PA-GFP-α-tubulin and RFP-H2B and arrested at metaphase with MG132 were identified by the RFP-H2B fluorescence. Microtubules adjacent to the metaphase plate on one side of the spindle structure were photo-activated with a 50 ms pulse from a 406 nm laser (60% power). Images were acquired using an Olympus 100X/1.35 lens with 200 ms exposures using the adaptive intervals program in SoftWorx (Applied Precision). Fluorescence intensities were measured with FIJI (http://fiji.sc/Fiji) and normalised to the background. Microtubule turnover half-lives for each cell were calculated by fitting a double-exponential curve as described in (Samora and McAinsh, 2011). No image processing technique was used in these analyses.

2.4.*In vitro* transcription, microinjections, live oocyte imaging and quantitative analysis

GFP-β-Tubulin, Histone H2B-RFP, GFP-ALADIN mRNAs were synthesised with the T3 mMessage mMachine Kit (Ambion) according to the manufacturer's instructions, and purified using LiCl Precipitation (Ambion) or RNAeasy columns (Qiagen). The plasmids pRN3 GFP-Tubulin, Histone H2B-RFP were a gift from K. Wassmann (CNRS, Paris, France) (Touati et al., 2012). Human GFP-ALADIN was cloned into pRN3E (gift from K. Wassmann) with XhoI and BamHI to obtain GFP N-terminus-tagged ALADIN.

Oocytes arrested in prophase were injected using a FemtoJet microinjector (Eppendorf) with constant flow settings. To allow expression of fusion proteins and oocyte recovery, oocytes were incubated for 3-6 h in media supplemented with milirone. After release into M2 medium, oocytes were imaged every 30 min for up to 30 h on an inverted Nikon TE2000E microscope with a Plan APO 40×/1.25 NA objective or Plan APO 63×/1.4 NA objective, a CoolSNAP HQ camera (Photometrics), standard filter sets and with an environmental chamber to maintain 37° C. Z-series optical sections of 80 µm were recorded with slices taken every 7 µm. For analysis of Polar body

extrusion a Plan APO 20×/0.75 NA objective was used. All images were acquired and analysed using the Nikon NIS-Elements ND2 software.

Quantitative analysis of time-lapse data was analysed manually using no processed images. Metaphase I time was defined when bipolar spindles structures have the most compact and organised metaphase plate. At this stage, pole to pole length of the spindle was measured using the tubulin signal. To calculate the migration of the spindle to the cortex, the time when one of the spindle pole reached the oocyte cortex was compared to the corresponding metaphase I timing. At this time, the angle made by the converging spindle pole with the cortex and spindle structure was measured.

2.5.Immunofluorescence microscopy, quantitative analysis and antibodies used

The following antibodies were used for immunofluorescence (IF) and western blot (WB) studies: rat anti-α-tubulin at 1:200 for IF (Sigma, YOL1/34), mouse anti-α-tubulin at 1:1000 for WB (Sigma, DM1A), human anti-centromere antibodies CREST at 1:1000 for IF (Fitzgerald), rabbit anti-Calnexin 1:75 for IF (Cell Signaling, C5C9), rabbit anti-Kif2a at 1:1000 for IF (gift from D.A. Compton, School of Medicine at Dartmouth, USA), rabbit anti-Ska3 at 1:500 for IF (gift from A. SantaMaria, University of Basel, Basel, Switzerland), rabbit anti-HAUS6 at 1:200 for IF (gift from, L. Pelletier, Lunenfeld Research Institute, Canada). Rabbit anti-phospho-S55-Hec-1 at 1:500 (GeneTex, GTX70017), mouse and rabbit anti-pericentrin at 1:500 (Abcam, mAbcam 28144) and 1:1000 (Abcam, ab4448) for IF and mouse anti-plk1 at 1:1000 for IF (Merck Milipore, clone 35-206, 32157), all gifts from J. Swedlow, University of Dundee. Rabbit anti-CEP192 1:200 for IF (Bethyl, A302-324A) and 1:2000 for WB (Novus Biologicals, NBP-84634) and mouse anti-NEDD1 1:1000 for IF (Abnova, 561-660), all gifts from S. Rocha, University of Dundee. Rabbit anti-ALADIN 1:1000 for blotting and 1:200 for IF (Proteintech Europe, 15127-1-AP), mouse anti-ALADIN at 1:100 for IF (monoclonal B-11, sc-374073, Santa Cruz), rabbit anti-NuMA at 1:1000 for IF and 1:2000 for blotting (NB-500-174), rabbit anti-Tpx2 at 1:1000 for IF (NB500-179), rabbit anti-Eg5 at 1:1000 for IF (NB500-181), all from Novus Biologicals. Rabbit

anti-Aurora A at 1:1000 for IF (ab12875-100), rabbit anti-phospho T288 Aurora A at 1:400 for IF (ab58494), rabbit anti-Adracalin (ALADIN) at 1:200 for IF (ab152122 – only for cilia studies using methanol fixation), rabbit anti-HURP at 1:500 for IF (ab70744) and mouse anti-Tacc3 at 1:1000 for IF (ab56595), all from Abcam. For western blot studies, Rabbit anti-Aurora A was used at 1:1000 (BioLegend, Poly6033). When necessary, GFP-fusion protein signals were enhanced in IF with GFP-booster 1:200 (Chromotek) or sheep anti-GFP antibody at 1:200 (BD Biosciences).

Secondary antibodies were highly cross-subtracted Alexa Fluor 488, 555, 594, and 647 conjugated anti-mouse, -human, -rabbit, -sheep and -rat and used at 1:500 or 1:200 (Life Technologies) for IF. Cross-subtracted DyLight 488 and Cy3 conjugated Donkey anti-rat and anti-mouse IgG (Jackson ImmunoResearch) were used at 1:500 for IF. 4',6-diamidino-2-phenylindole (DAPI) was used at 2 μg/ml. For western blotting, primary antibodies were detected with either HRP conjugated secondaries diluted 1:5000 (Promega) and developed with ECL (GE Healthcare) or with IR conjugated secondaries (IRDye800, Rockland Immunochemicals or Alexa Fluor-680, Life Technologies) diluted 1:5000 and visualised on a LI-COR Odyssey (LI-COR Biosciences).

2.5.1.Human cells

Cells grown on 22x22 mm #1.5 coverslips were fixed with room temperature 4% formaldehyde (Electron Microscopy Sciences) in 1x PHEM (60 mM PIPES, 25 mM HEPES, 10 mM EGTA and 2 mM $MgCl_2$ pH 6.9) for 5 min, followed by 5 min permeabilisation with PHEM + 0.5% Triton X-100 (Roche) and then fixed again. After fixation and permeabilisation, coverslips were incubated for 5 min in PHEM + 0.1% Triton X-100 (PHEM-Wash), TBS+ 0.1% Triton X-100 and again in PHEM-Wash. Blocking and antibody dilution buffer was TBS, 0.1% Triton X-100, 2% BSA, 0.1% Sodium Azide. Coverslips were blocked for 30 min at room temperature (25ºC), primary antibodies incubation were done overnight at 4ºC, and secondary antibody incubations were done at room temperature for 2 h. Excess antibodies were removed after primary and secondary antibody incubations by three washes with PHEM–Wash for 5 min. Coverslips were mounted in Dako Fluorescence Mounting Medium (Dako) and sealed using CoverGrip

Coversplip Sealant (Biotium). To assay for k-fibre microtubule stability, cells were placed for 10-20 min on a solid aluminium block pre-chilled and resting on ice. Then, cells were fixed in pre-chilled 90% methanol, 1.6% formaldehyde, 5 mM Sodium bicarbonate pH 9 for 10 min at - 20º C.

Fixed cells were imaged on a Delta-Vision Core microscope (Applied Precision) with a 100x oil-immersion 1.35 N.A. objective lens (Olympus), a CoolSNAP HQ camera (Photometrics) and standard filter sets. Three-dimensional images were acquired with the SoftWoRx software (version 5.5.0; Applied Precision) and Z-series optical sections were recorded with slices taken every 0.2 μm.

Quantitative analysis of fluorescence microscopy data was performed using OMERO software (Open Microscopy Environment (Allan, 2012)) and OMERO.mtools a MATLAB (Mathworks) plugin (http://www.openmicroscopy.org/site/products/partner/omero.mtools).

For the analysis, DeltaVision files were imported into OMERO and masks of regions of interest were created by defining the Otsu threshold of the α-tubulin signal (spindle mask) or the pericentrin signal (centrosome mask). Measurements of average intensity/pixel across Z series for each experiment were calculated in each channel within the mask regions defined. Relative mean intensity values or pixel numbers were obtained after correcting for the background fluorescence. For spindle-length measurements, pole-pole distance was measured using OMERO and α-tubulin signal. To measure spindle pole focusing, angles made by converging spindle microtubules were measured manually for each pole stained for tubulin using FIJI. To measure kinetochore-kinetochore distances, single image planes were selected where kinetochore pairs were clearly resolved, and the distance between them were measured with OMERO. The angles of the line connecting the kinetochore pair made with respect to the metaphase plate were measured with OMERO after rotated the images of the spindles so that the metaphase plate was horizontal and perpendicular to the centrosomes. To quantify chromosome alignment, the volume of the metaphase plate of each cell, the pixel area across Z series was calculated using the Otsu threshold

of the DAPI signal using OMERO.mtools. No image processing technique was used in these analyses.

All images were stored in OMERO and figures generated using OMERO.figure (http://figure.openmicroscopy.org), FIJI or Photoshop (Adobe).

2.5.2.Oocytes

Oocytes at the indicated maturation stage were briefly exposed to acid Tyrode's solution to partially remove the *zona pellucida* and excess of acid removed by pre-warm M2 medium. Then, oocytes were transferred to PHEM and incubated in 0.1% Triton X-100, 0.2% Glutaraldehyde, 2% PFA in PHEM 1x for 14 h at 4º C. After incubation, oocytes were transferred to Superfrost Plus Microscope Slides (Fisher Scientific) and let them attached at room temperature (25º C) without drying out by adding excess of PHEM. Oocytes were moved into a small region defined previously using nail polish to control the surface tension. After three washes in PHEM-Wash, 10 min each, oocytes were incubated in 1% Triton 1x PHEM for 30 min, followed by 3% BSA, 0.05% Tween 20 in PHEM 1x for 30 min also at room temperature (25º C). Using the same solution antibodies were diluted and oocytes incubated with primary antibodies in a humid chamber for overnight at 4º C, and secondary antibody at room temperature for 30 min. Excess antibodies were removed after primary and secondary antibody incubations by three washes with PHEM–Wash for 10 min. Coverslips were mounted in Dako Fluorescence Mounting Medium (Dako) and sealed using nail polish.

Fixed oocytes were imaged in a Leica SP2 confocal microscope and images processed in FIJI.

2.6.Immunoblotting and Immunoprecipitation

G2/M arrested cells were obtained by mitotic shake off. Mitotic enriched extracts or whole-cell extracts were prepared by lysing cells in 10 mM Tris- HCl pH 7.5, 500 mM NaCl, 0.5 mM EDTA, 0.5% NP-40, 0.25 mM Pefablock (Sigma) and EDTA-free complete protease inhibitor mix (Roche). When to analyse phopho-proteins, 1x Phospho STOP (Roche) was added to the lysis buffer and washing solutions.

For immunoprecipitation, lysates were cleared by a pre-spin at 20,000 x g, 5 min at 4º C, pre-equilibrated in 20 mM HEPES pH 7.9. 0.5 mM DTT and 20% Glycerol, incubated for 16 h at 4º C with anti-ALADIN, anti-GFP, or the appropriate control IgG (Sigma), followed by 2 h incubation with rotation with protein G-sepharose beads at 4º C. Beads were washed 3 times with cold PBS buffer and bound proteins were eluted in 2X LDS-sample buffer (NuPAGE, Life Technologies).

Immunoprecipitated proteins or cell-extracts were separated on NuPAGE 4-12% Bis-Tris Gels (Life Technologies) and then transferred to nitrocellulose membranes (Whatman) and westerns were performed as described (Griffis et al., 2007).

2.7.Chromosome spreads

Chromosome spreads were prepared according to the adapted method in (Revenkova et al., 2010). Brief, *zona pellucida* of oocytes arrested at metaphase II with polar body was removed and oocytes were incubated in 0.75% sodium citrate solution and then fixed in 4% PFA in PBS. After drying at room temperature in a humidified chamber, chromosomes were stained with Hoechst 33342 (1µg/ml in PBS; Sigma) and mounted with Vectashield (Vector Laboratories, Burlingame, CA).

2.8.Conventional and Laser-assisted *In Vitro* Fertilisation (IVF)

For conventional IVF, ampulla from super ovulation mice were dissected from the oviduct and the cumulus-oocyte-complexes released, and incubated with fresh spermatozoa during 4 h at 37° C with humidity control 5% CO_2 atmosphere in HTF (Human Tubal Fluid) medium. After incubation, oocytes were washed into KSOM mouse embryo culture medium to remove spermatozoa and cumulus cells and the fertilisation status of each oocyte was assessed by the presence of pronuclei 10 h after the beginning of the insemination. All oocytes were washed and transferred to pre-equilibrated KSOM medium and incubated at 37º C. Concentration and motility of fresh spermatozoa was evaluated and only spermatozoa with standard qualities were used.

After overnight culture of the oocytes, 2-cell stage embryos were transfer into KSOM and their development monitored.

For laser assisted IVF, oocytes from *in vitro* maturation were perforated at room temperature on a Zeiss Microscope Axiovert 200M equipped with the laser set at 63% power and 600 µs. Perforations took approximately 10 min per conditions. Perforated oocytes were washed and transfer into an IVF drop containing spermatozoa, as described above.

2.9.Statistical analysis and data representation

Data obtained from Omero, FIJI and NIS-Elements software was imported into Sigma-plot software (Systat Software Inc), which was used to generate box-and-whisker, polar and cumulative distribution plots. In box-and- whisker plots the middle line shows the median value; the bottom and top of the box show the lower and upper quartiles; whiskers extend to 10th and 90th percentiles, and all outliers are shown. Student's t-test was used to determine statistical significance between two treatments, and for evaluating three or more data sets one-way analysis of variance was utilised (significance is reported on each figure).

3.Characterisation of ALADIN's function during mitosis

The majority of the results described in this chapter have been already published (Carvalhal et al., 2015).

3.1.Introduction

The nuclear pore complex (NPC) mediates the bidirectional exchange of macromolecules across the nuclear envelope. NPC is a large protein complex composed of multiple copies of approximately 30 different proteins called nucleoporins (NUPs) (Raices and D'Angelo, 2012).

Higher eukaryotes undergo a dramatic reorganisation as the cell prepares to segregate the duplicated chromosomes during mitosis. An important element of cell division is the assembly of the mitotic spindle. At prophase, the spindle formation begins after nuclear envelope breakdown (NEBD) when microtubules start to nucleate from each centrosome. During NEBD, NPCs disassemble into sub-complexes or even single NUPs, which are redistributed within the mitotic cytoplasm or associated with mitotic structures (Antonin et al., 2008; Kahms et al., 2011). Recent studies have shown that they are not inert during this process; some of them actively participate in the faithful chromosome segregation (Imamoto and Funakoshi, 2012). NUPs are essential for proper kinetochore and spindle function, spindle assembly checkpoint signalling, NEBD and reassembly (Arnaoutov and Dasso, 2003; Babu et al., 2003; Belgareh et al., 2001; Cross and Powers, 2011; Itoh et al., 2013; Klein et al., 2009; Lee et al., 2008; Loiodice et al., 2004; Platani et al., 2009; Prunuske et al., 2006; Rasala et al., 2008).

The alignment of chromosomes from prophase to metaphase as well as their accurate segregation to daughter cells at anaphase involves a highly regulated rearrangement of the entire cell. Such dramatic changes are regulated by a complex network of mitotic kinases, which regulates hundreds of different spindle substrates to modify their activities and localisation (Nigg, 2001). One of these is the Aurora A kinase that resides at centrosomes and spindle poles, and is required at multiple steps during mitosis (Carmena et al., 2009). Perturbation of Aurora A affects mitotic entry, centrosome maturation, bipolar spindle assembly, and anaphase microtubule stability (Asteriti et al., 2011; Barr and Gergely, 2007; Lioutas and Vernos, 2014). Aurora A is a serine/threonine kinase highly regulated by phosphorylation in a cell cycle dependent manner. Its catalytic activity is dependent on the autophosphorylation on Threonine 288, which is mediated by Tpx2 or CEP192 (Bayliss et al., 2003; Joukov et al., 2010; Joukov et al., 2014; Kufer et al.,

2002). Key cofactors such as Ajuba, Bora, PAK, protein phosphatase inhibitor-2, and nucleophosmin also contribute to the regulation of Aurora A's activity and/or localisation (Hirota et al., 2003; Hutterer et al., 2006; Reboutier et al., 2012; Satinover et al., 2004; Zhao et al., 2005; Zorba et al., 2014)

Here it is described for first time the participation of the nucleoporin ALADIN in mitosis, in particular in spindle formation and the spatial regulation of Aurora A at spindle poles. In 2002, ALADIN was identified as a component of the NPC (Cronshaw et al., 2002), but it was first associated with triple A syndrome (Huebner et al., 2000; Tullio-Pelet et al., 2000). Triple A syndrome is a complex and multisystemic autosomal-recessive disease that manifests in a triad of ACTH-resistant adrenal insufficiency, achalasia and alacrima symptoms accompanied by neurological impairment. This disease is caused by mutations in the gene that encodes the protein ALADIN but no genotype-phenotype correlation has been identified so far (Prasad et al., 2014; Sarathi and Shah, 2010). Here it is also shown that mitotic phenotypes observed after ALADIN depletion also occur in cells from triple A syndrome patients, which raises the possibility that mitotic errors may underlie part of the etiology of this syndrome.

3.2. ALADIN depletion perturbs spindle formation

Several NUPS have been shown to be involved in mitosis (Chatel and Fahrenkrog, 2011). To identify new NUPs during this process, *Drosophila* S2 cells stably expressing GFP-H2B and mCherry-α-tubulin were treated with dsRNAs for each nucleoporin and imaged with an automated microscope once per minute over the course of two hours. Using this comprehensive screen, my laboratory has identified that ALADIN is a new regulator of mitosis in *Drosophila* S2 cells (Figure 16). It was observed that depletion of ALADIN from S2 cells strikingly delayed spindle formation but anaphase would still initiate (Figure 16A and B; ***p<0.003).

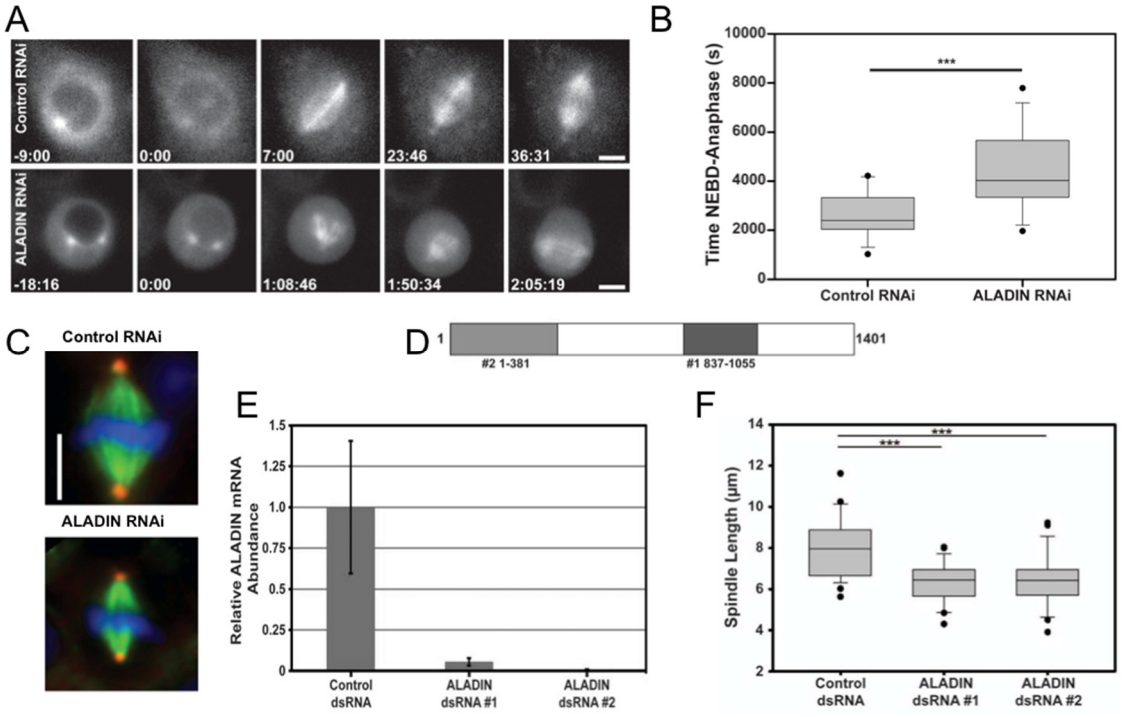


Figure 16: Depletion of ALADIN from *Drosophila* S2 cells impairs spindle assembly and produces shorter spindles.
(A) **Representative images of cells stably expressing mCherry-α-Tubulin treated with control or ALADIN specific dsRNA (RNAi) during spindle assembly. Time 0:00 represents NEBD. (B) ALADIN depletion slows spindle formation. Quantification of the time between NEBD and anaphase of 15 control cells and 12 ALADIN depleted cells. The triple asterisk indicates p<0.003. (C) Representative images of fixed S2 cells fixed and stained to visualise α-tubulin (green), γ-tubulin (red) and DNA (blue). (D) Schematic diagram of the regions of ALADIN mRNA target by independent oligos – ALADIN dsRNA#1 between the region 837 and 1055. ALADIN dsRNA#2 for the region 1 until 381. (E) Levels of ALADIN mRNA levels after treatment with both dsRNAs. (F) Quantification of pole-pole distance in control and ALADIN-silenced cells with both dsRNAs. At least 24 spindles measured in each condition. ***p<0.001. Scale bar=5µm. Box-and-whisker plot: middle line shows the median value; the bottom and top of the box show the lower and upper quartiles; whiskers extend to 10th and 90th percentiles, and all outliers are shown. Bar chart: Mean ± standard deviation. (A) – (C) provided by E. Griffis. (E) provided by T. Kasciukovic.**

The spindle morphology of ALADIN depleted cells appeared to be largely normal (Figure 16C), but spindles were significantly shorter when compared with control dsRNA treated cells. To confirm the specificity of this phenotype, cells were treated with two non-overlapping oligos, Figure 16D, and spindle lengths measured in fixed samples. Both oligos effectively decreased ALADIN mRNA levels (Figure 16E) and produced short spindles (Figure 16F, ***$p<0.001$).

These results suggest that ALADIN is a new regulator of mitosis, however previous studies by Goshima and collaborators in the *Drosophila* genome failed to identify ALADIN as a new regulator due to their shorter RNAi treatment protocol, which likely failed to reduce spindle length to a point where it could be detected by an automated analysis program used to scored spindle phenotypes (Goshima et al., 2007).

3.3.ALADIN localises around the mitotic spindle and at the spindle poles in *Drosophila* and human cells

ALADIN's localisation during mitosis has never been described. Therefore, to address its localisation, S2 cells stably expressing GFP-ALADIN and mCherry-α-tubulin were used. During interphase, *Drosophila* GFP-ALADIN is localised at the nuclear envelope (Figure 17A, upper panel) in agreement with previous observations of ALADIN's localisation in human cell lines (Cronshaw and Matunis, 2003).

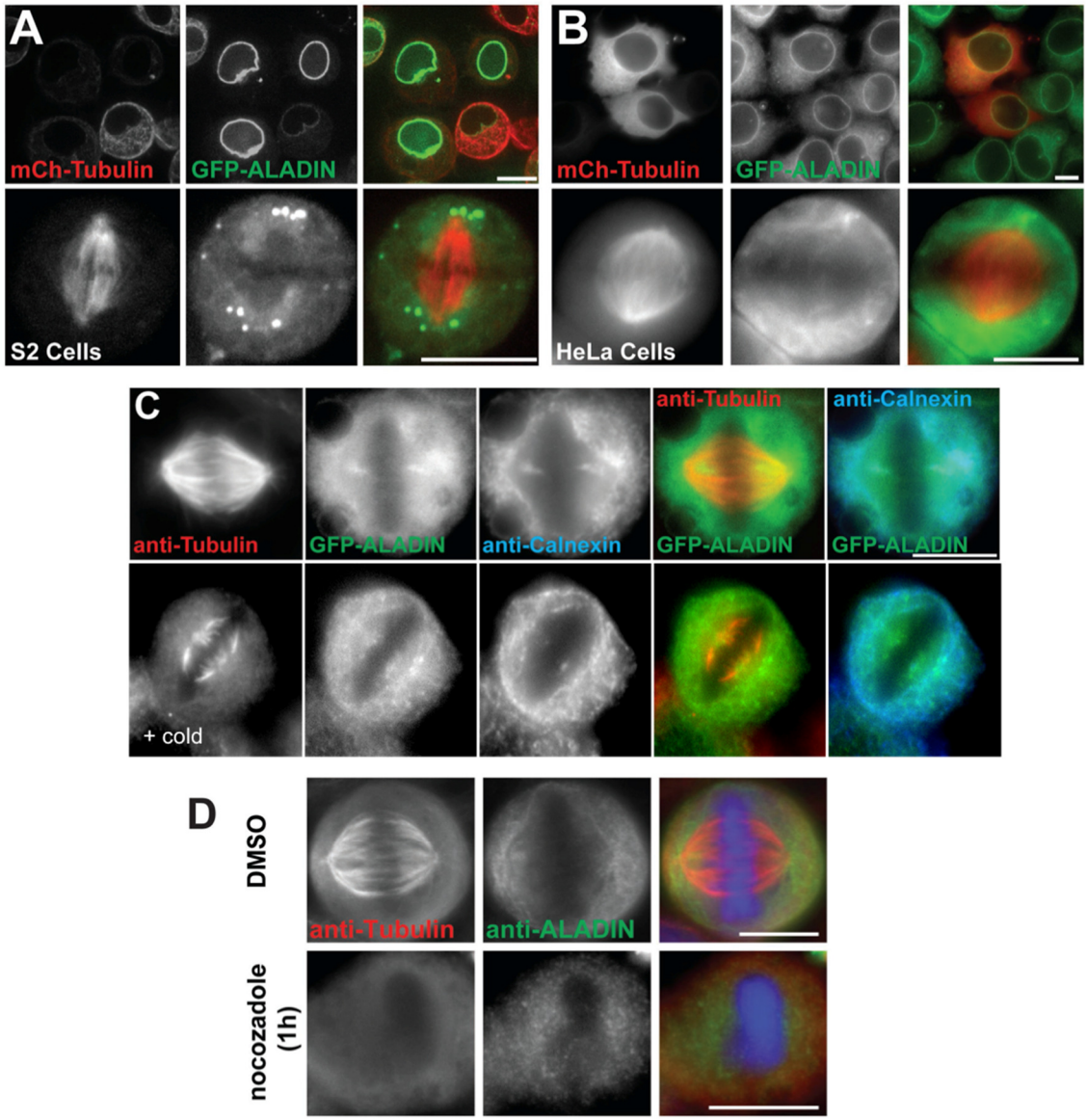


Figure 17: ALADIN localises around the mitotic spindle and at the spindle poles in Drosophila and human cells.
(A) Representative images of *Drosophila* S2 cells stably expressing GFP-ALADIN and mCherry-α-tubulin in interphase (upper panel) or metaphase (bottom). (B) HeLa cells stably expressing GFP-

ALADIN and transiently transfected with mCherry-α-tubulin in interphase (upper panel) or metaphase (bottom). (C) GFP-ALADIN partially colocalises with Calnexin during metaphase but not after cold treat cells during 20 minutes on ice. Representative images of cold methanol fixed HeLa cells stably expressing GFP-ALADIN are shown. (D) HeLa cells were fixed in presence or absence of nocodazole and then stained to visualise α-tubulin (red) and endogenous ALADIN (green). Scale bars are 10 µm in A and B and 5 µm on remaining images. (A) and (B) provided by E. Griffis.

Surprisingly, at metaphase, ALADIN did not co-localise on kinetochores or discrete k-fibre bundles as it was been described for other NUPs, (Cronshaw and Matunis, 2003); instead, ALADIN localised diffusely throughout the spindle, where it was excluded from chromatin but more enriched around the spindle (Figure 17A, lower panel).

Next, ALADIN's localisation during metaphase was addressed in human cells. During interphase, as expected, HeLa cells stably expressing GFP-ALADIN had their GFP signal localised at the nuclear envelope (Figure 17B, upper panel). At metaphase, GFP-ALADIN was localised diffusely throughout the spindle (Figure 17B, lower panel), as in *Drosophila* cells. Endogenous staining of this protein in HeLa cells also showed some accumulation at the spindle poles, where the highest concentration appeared between the centrosome and metaphase plate (Figure 17C and D).

The Golgi apparatus and endoplasmic reticulum (ER) during mitosis are localised around the spindle at metaphase (Jesch and Linstedt, 1998; McCullough and Lucocq, 2005). To test whether ALADIN co-localises with the mitotic ER remnant, GFP-ALADIN expressing cells were fixed and stained with an antibody that recognises calnexin, an integral ER protein (Figure 17C). The high concentration of ALADIN that is proximal to the spindle pole co-localised with calnexin and is therefore likely to be an ER membrane-associated pool of the protein. Next, it was determined if ALADIN's mitotic localisation was dependent on microtubules using cells treated with cold to preserve only the microtubules bundles of k-fibres (Figure 17C, lower panel), or nocodazole to totally depolymerise the microtubules (Figure 17D). After cold treatment, ALADIN's localisation became more enriched at the spindle pole, despite a lack of the co-recruitment of calnexin. ALADIN's localisation was totally lost after total depolymerisation of microtubules with nocodazole.

3.4.ALADIN is required for proper spindle assembly in human cells

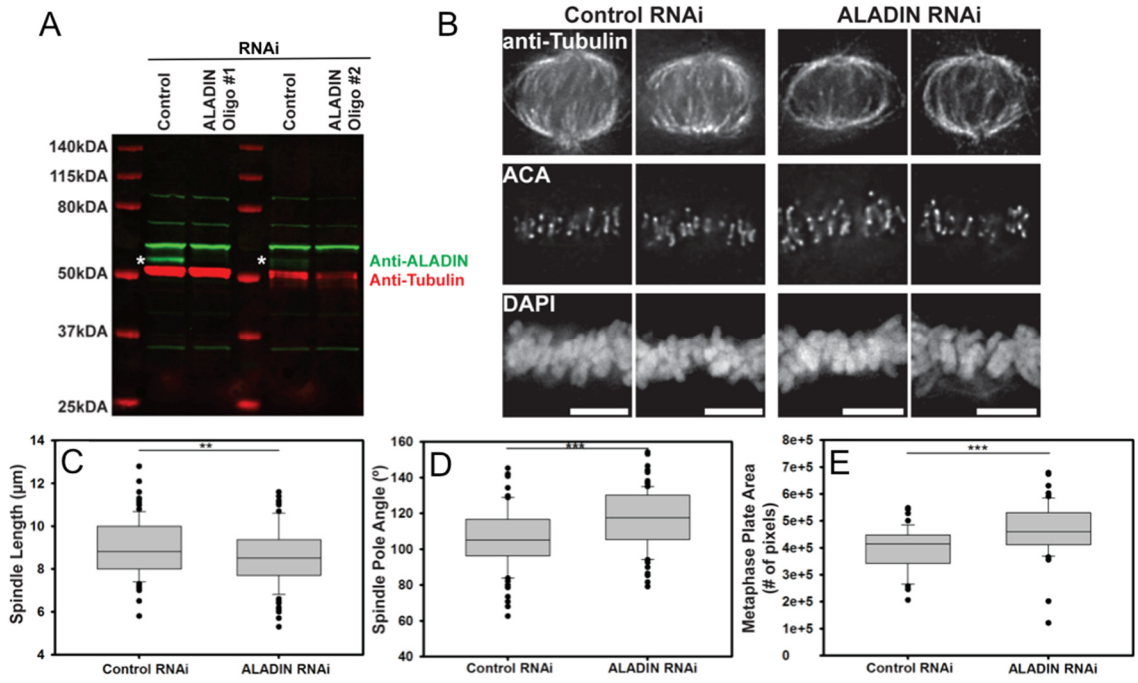


Figure 18: ALADIN is required for proper spindle morphology.
(A) Effective depletion of ALADIN by two non-overlapping oligos specific for human ALADIN. Tubulin was used as loading control (red). The anti-ALADIN antibody recognises non-specific cross-reacting bands, asterisks indicate ALADIN specific band (green). (B) Representative images of fixed HeLa cells after treatment with control and ALADIN siRNAs and MG132 for four hours. Cells were stained for microtubules (α-tubulin), kinetochores (ACA) and chromosomes (DAPI). Scale bars=5µm. (C) ALADIN silencing reduces spindle length in metaphase (8.983µm *vs* 8.560µm). Quantification of at least 100 pole-pole distances of cells treated with the indicated siRNAs, fixed and stained for α-tubulin. n=4. (D) Quantification of the angle made by converging spindle microtubules at the pole of at least 55 cells in each conditions from three independent replicates (106.0˚ *vs* 117.3˚). (E) The area of DAPI signal across Z-stacks was measured for metaphase plates of fixed cells treated with the indicated siRNAs. More than 55 cells analysed from three biological replicates. Box-and-whisker plots: middle line shows the median value; the bottom and top of the box show the lower and upper quartiles; whiskers extend to 10th and 90th percentiles, and all outliers are shown. **p<0.05 and ***p<0.001.

Since ALADIN's localisation is conserved, it was next examined whether ALADIN also has a role in mammalian mitosis, and so, HeLa cells were treated with siRNAs that target human ALADIN (two non-overlapping oligos were used, and around 90% depletion of the endogenous protein was obtained, Figure 18A). To synchronise control and ALADIN-specific siRNA-treated cells in metaphase, cells were treated with MG132 for four hours. Representative images of fixed cells stained to visualise microtubules, kinetochores, and DNA in both conditions are shown in Figure 18B.

As in ALADIN dsRNA-transfected S2 cells, depletion of ALADIN in HeLa cells also led to shorter spindles (5% shorter than control spindles; Figure 18C; **p< 0.05). Additionally, it was observed that the angle made by converging spindle microtubules at the pole was greater in the ALADIN-depleted cells (10% increase, ***p<0.0001, Figure 18D); suggesting a decrease of pole-focusing in absence of ALADIN. Looking at the metaphase plate, ALADIN depleted cells appeared to have disorganised chromosomes (Figure 18B). Therefore, the volume of DAPI signal for each cell was quantified and ALADIN-depleted cells showed a significant increase in chromatin area compared with control conditions (***p<0.001; Figure 18E).

Next it was decided to test whether human ALADIN is also required for proper timing of spindle assembly (Figure 19A). For that, cells were treated overnight with monastrol to generate monopolar spindles. Thirty minutes after monastrol washout, both control and ALADIN depleted cells were predominantly found to be in prophase-prometaphase. After 45 minutes, control cells showed a reduction in prophase-prometaphase cells and an increase in anaphase-telophase cells; the ALADIN-depleted cells however, did not show such reduction in prophase-prometaphase cells until the 60 minute time point; suggesting that ALADIN depletion, as in *Drosophila*, also slows spindle assembly in human cells. In agreement with that, it was observed that ALADIN-depleted cells arrested in mitosis with MG132 had more cells with Mad2-positive kinetochores (indicating unaligned kinetochores) and more Mad2-positive kinetochores per cell (supporting the delayed chromosome alignment during mitosis) (Figure 19B; ***p<0.05).

To further characterise the consequences of ALADIN depletion in mitosis, spindle microtubules were analysed. Given that microtubules that attach to kinetochores become bundled into k-fibres and are more stable than the populations of interpolar microtubules (Samora and McAinsh, 2011), cells were exposed to cold to assay k-fibre stability. Cold-treated ALADIN depleted cells showed a marked reduction of stable k-fibres microtubules (Figure 19C) when compared with control cells. Nonetheless, this reduction on k-fibres stability, after ALADIN silencing, did not represent an overall reduction in spindle microtubules, as the average tubulin intensity was slightly but not statistically significantly increased relative to control cells (Figure 19D). So, it was next tested whether k-fibres were destabilised as a consequence of an increase in the overall dynamics (or

flux) of microtubules within k-fibres. A HeLa cell line stably expressing photoactivatable-GFP-α-tubulin (PA-GFP-tubulin) was treated for the both siRNA conditions and after photo-activating a spot within the spindle, the reduction in the fluorescence of PA-GFP-tubulin was followed through time. Representative plots are shown in Figure 19G. By fitting a double exponential decay (Bakhoum et al., 2009; Samora and McAinsh, 2011) the half-time of microtubule turnover for the fast (interpolar microtubules, Figure 19E) and slow populations (k-fibres, Figure 19F) were determined. It was observed that ALADIN silencing induced a slightly slower flux of interpolar microtubules ($p=0.123$) and faster flux of k-fibre microtubules ($p=0.957$), however neither of these changes were statistically significant. Therefore, faster rates of microtubule depolymerisation can be excluded as being a major factor leading to the destabilisation of k-fibres after ALADIN depletion.

Since k-fibres are microtubule bundles that attach sister kinetochores to spindle poles, so it was investigated if the destabilisation of k-fibres observed after ALADIN depletion could affect pulling forces at the kinetochores. Therefore, (i) the angle distribution of kinetochore pairs relative to the metaphase plate and (ii) the interkinetochore distance were measured in control and ALADIN siRNA treated cells. Depletion of ALADIN caused a significant 14% increase in interkinetochore stretch (D; $p < 0.001$), and a greater spread (θ) of kinetochore pair angles (Figure 19H). Nevertheless, the kinetochore structure did not appear to be grossly affected by ALADIN silencing as staining of kinetochore and centromere proteins (Hec1, Ska3, and Sgo1) nor visualisation of MCAK in control and ALADIN-silenced cells were not different (Figure 20).

Altogether, these results suggest that ALADIN is important for proper mitosis by controlling spindle stability but without compromising kinetochore-microtubule binding.

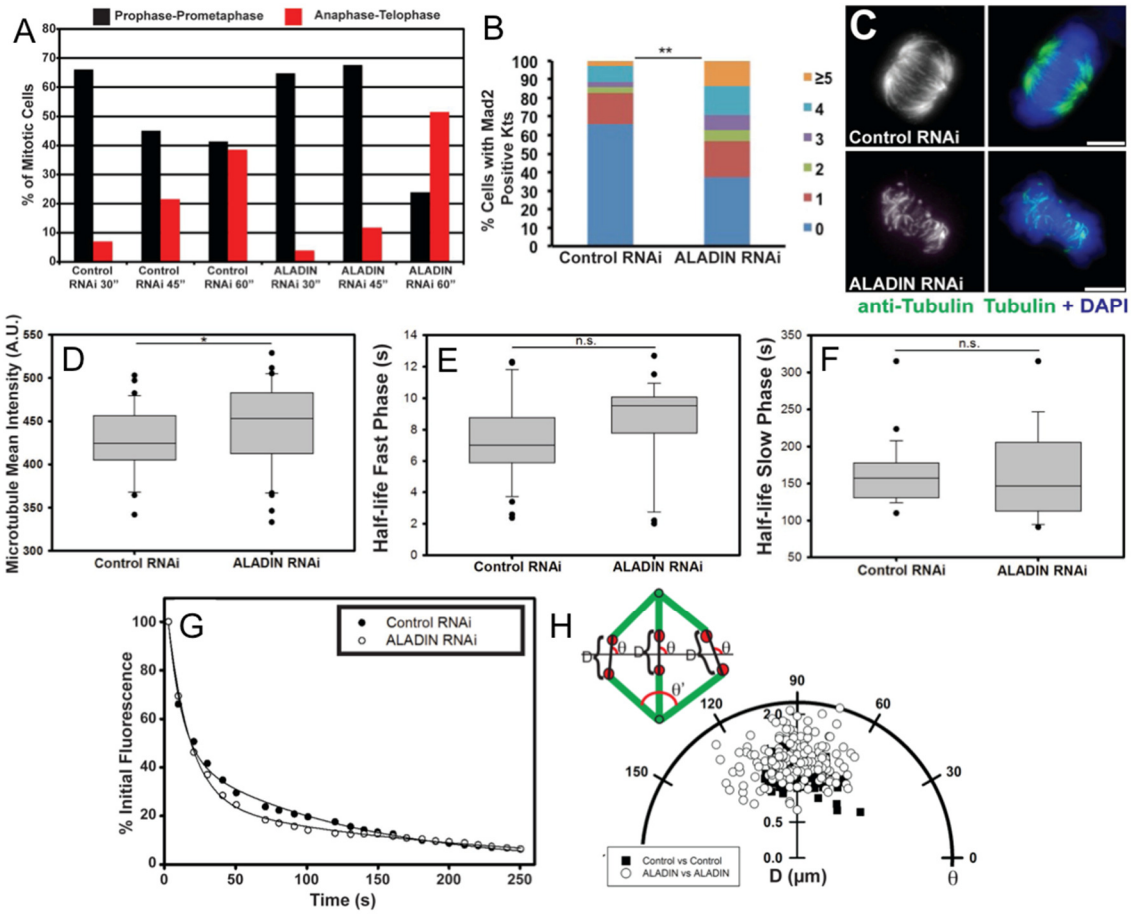


Figure 19: ALADIN is required for timely chromosome alignment and k-fibre stability.
(A) Control and ALADIN-silenced cells were treated overnight with monastrol to generate monopolar spindles. Cells were fixed at 30, 45 and 60 minutes after washout the inhibitor, and stained cells to visualise microtubules and DNA. Images of these samples were acquired and individual cells were scored for their mitotic state into three subclasses: prophase-prometaphase, metaphase and anaphase-telophase. Percentage of cells in each subclass is plotted for the indicated time points. (B) Control and ALADIN-depleted cells arrested in mitosis for four hours with MG132 were fixed and stained for Mad2 and ACA. The percentage of cells from two independent experiments with 1,2,3,4 or 5 or more Mad2 positive kinetochore pairs is presented. (C) Representative maximum intensity projection of cold-treated cells with depletion of ALADIN were fixed and stained for microtubules (green) and chromosomes (DAPI). Scale bars= 5 µm. (D) ALADIN-silenced cells does not affect microtubule intensity. Mean intensity of at least 40 spindles was measured for the indicated siRNAs, n=3. The half-time of the fast (E) and (F) slow-phase of microtubules was measured using PA–GFP–tubulin cells. By fitting a double exponential decay to the reduction of the fluorescence after photo-activation (representative decay shown in (G)) in at least 24 cells per condition, it was extracted the half-time of microtubule turnover for interpolar microtubules (Fast-phase: 7.395s *vs* 8.502s, p=0.123) or k-fibres (Slow-phase: 161.02s *vs* 160.21s, p=0.957). n=3 (H) Polar plot of the angle (θ) of the line connecting the kinetochore pair and the distance (D) between the outer edges of kinetochore pairs with respect to the metaphase plate. Acquired images were rotated in order to have the metaphase plate horizontal and perpendicular to the centrosomes. Quantification from at least 200 kinetochore pairs. Box-and-whisker plot: middle line shows the median value; the bottom and top of the box show the lower and upper quartiles (25-75%); whiskers extend to 10th and 90th percentiles, and all outliers are shown. *p<0.1, **p<0.05 and n.s= no statistically significant after performed a t-test. (A) and (H) provided by E. Griffis.

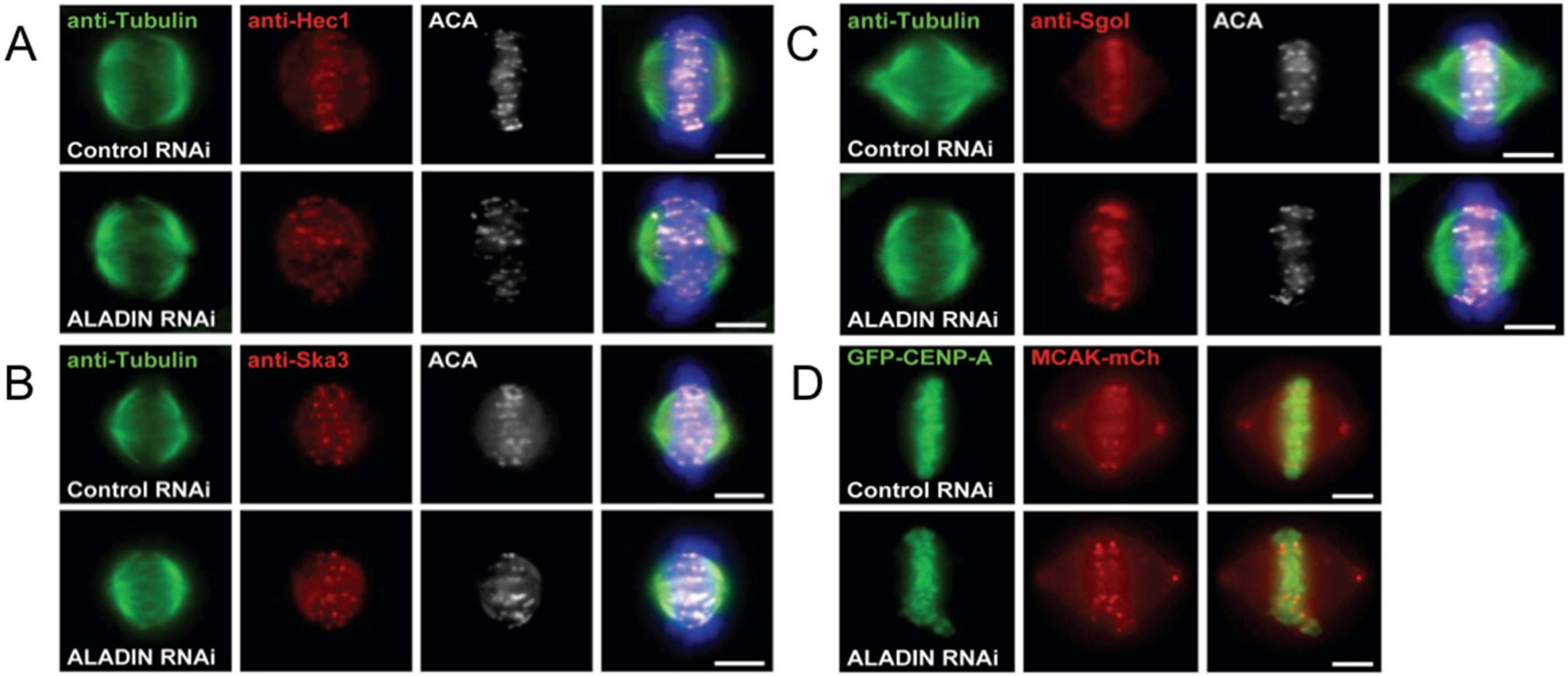


Figure 20: Kinetochore and centromeric proteins are not affected after ALADIN depletion.
(A-C) Representative Z-stacks of control or ALADIN-silenced HeLa cells arrested in metaphase for four hours and fixed and stained for the indicated antibodies. (D) Representative live-cell images of control or ALADIN siRNA-transfected HeLa cells stably expressing MCAK-mCherry and GFP-CENP-A. Scale bars= 5µm.

3.5.ALADIN is essential for localising Aurora A to spindle poles

Mitotic kinases are crucial regulators of multiple aspects of spindle function (Nigg, 2001). To gain more mechanistic insight into ALADIN's mitotic role, the localisation of major kinases involved in the process was addressed after ALADIN depletion.

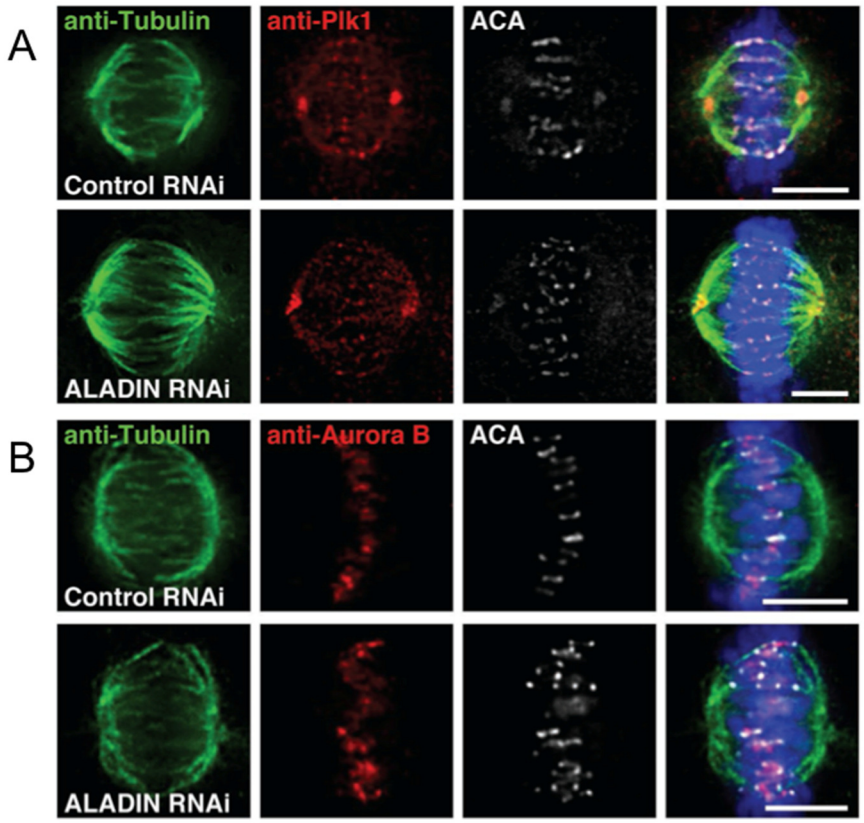


Figure 21: Localisation of Aurora B and Plk1 are unaffected after ALADIN depletion. Representative deconvolved Z-series images of cells treated with control or ALADIN-specific siRNAs and fixed and stained with antibodies that recognise microtubules (tubulin, green), kinetochores (ACA, white) and the indicated kinases, (A) Plk1 or (B) Aurora B (both in red). Scale bars= 5µm.

Kinase activity at the kinetochores during mitosis is mainly orchestrated by Aurora B and Plk1 (Carmena et al., 2009; Nigg, 2001). Both Aurora B and Plk1's localisation were similar after control and ALADIN siRNA treatments (Figure 21). These findings are in line with the idea of normal kinetochore structure and functions under these conditions. However in cells lacking ALADIN, Aurora A, which has been implicated in several vital events in mitosis (Barr and Gergely, 2007; Lioutas and Vernos, 2014), was spread outwards from centrosomes and onto spindle pole microtubules, producing a 24% increase in the area of Aurora A positive pixels

(Figure 22A and B). Phosphorylation of the threonine 288 (pT288) within the activation loop is required for Aurora A kinase activity (Littlepage et al., 2002). By using a specific phospho-T288 antibody, the localisation of the only active Aurora A can be revealed. Because Aurora A accumulates at the centrosome but is also localised on the microtubule spindle pole (Lioutas and Vernos, 2014), the localisation of Aurora A in its active form (pT288) was firstly addressed at the centrosome. For that, pT288 levels were measured only in the region defined by the centrosome marker pericentrin, which is not changed after ALADIN silencing (Figure 22E and J). After ALADIN depletion, active Aurora A (pT288) was reduced by 38% at the centrosome when compared with control treated cells (***$p<0.001$; Figure 22C and D). Aurora A protein levels (measured by western blotting, Figure 22G) or total active Aurora A (total pT288 signal; Figure 22F) do not change after ALADIN depletion. These results suggest a misdistribution of active Aurora A at the centrosome and not an overall reduction of Aurora levels within the cell. Strikingly, overexpression of ALADIN (GFP-ALADIN) also diminished the intensity of active Aurora A (pT288) at the centrosome (Figure 22H and I, ***$p<0.001$). Full inhibition of Aurora A produces phenotypes that are much more extreme than it was observed after ALADIN depletion (Asteriti et al., 2011; Wang et al., 2008). Therefore, the ALADIN-depletion short spindle phenotype was compared with cells treated with a specific inhibitor of Aurora A activity, MLN8237. MLN8237 inhibition of Aurora A produced a more severe reduction in spindle length (35% reduction) than ALADIN depletion alone (Figure 22K), reinforcing the idea that ALADIN depletion only re-localises Aurora A. Also, the reduction in spindle length observed after MLN8237 treatment was not further augmented by ALADIN depletion.

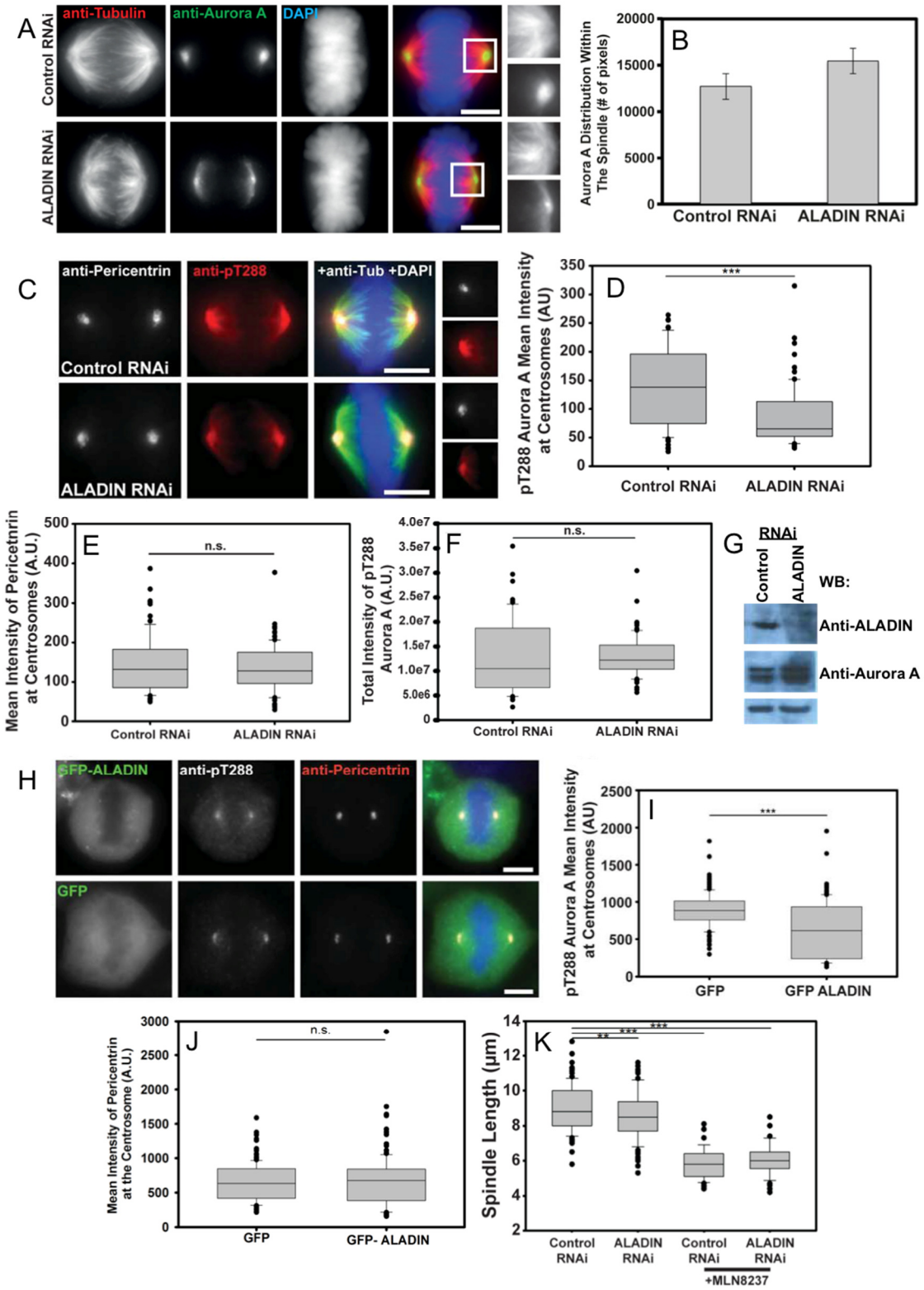


Figure 22: ALADIN is a novel regulator of Aurora A kinase.
(A) Representative maximum intensity projections of Aurora A (green), microtubules (red) and chromosomes (blue) in control and ALADIN-depleted cells are shown. For comparison purposes, magnified views of microtubules (top) and Aurora A (bottom) from within the boxed regions in the merged images are presented. (B) Quantification of the number of Aurora A positive pixels within the spindle is presented (12714 *vs* 15478; Mean ± SEM; at least 36 cells from four independent experiments. (C) Representative images of the localisation of active Aurora A at the spindle poles identified using the specific antibody phospho-T288 that only recognise Aurora A kinase on its active form. Magnified views are presented on the right side with microtubules on the top and active Aurora A on the bottom. (D) Average intensity of active Aurora A at the centrosome in cells treated with the indicated siRNAs. Pericentrin labeling was used to define the centrosome. Phospho-T288 intensity

was measured and restricted to only the centrosomal region. At least 39 cells were measured in each condition from two independent experiments. (E) Box-and-whisker plot showing that mean intensity for pericentrin does not change after ALADIN depletion (145.29 *vs* 134.71). More than 39 cells analysed in each conditions, n=2. (F) The total intensity of phospho-T288 Aurora A within cells treated with control or ALADIN specific siRNAs was measured in Z-stacks. At least 79 cells were measured for each condition for three independent experiments. p=0.822 (G) ALADIN depletion does not affect Aurora A protein levels. Whole cell lysates from cells treated with the indicated siRNAs were probed with anti-ALADIN, stripped and then with Aurora A antibody. Bottom panel shows unspecific bands used as loading control. See appendix for complete view of the blots used. (H) Representative images of pT288 labelling (Poser et al.) in HeLa cells transiently transfected for 48 hours with plasmids to overexpress GFP or GFP–ALADIN from a CMV promoter. (I) Box-and-whisker plot showing the mean intensity for active Aurora A (pT288) at the centrosome (892.219 *vs* 612.285), using pericentrin labelling to define the centrosomal region; which does not change after overexpression of ALADIN (J). At least 67 cells were measured in each condition, n=2. (K) Cells treated with the indicated siRNAs were incubated with or without MLN8237 and then fixed and stained to visualize α-tubulin and chromosomes. Spindle length was measured for more than 46 spindles for each condition across 3 independent experiments. Box-and-whisker plot: middle line shows the median value; the bottom and top of the box show the lower and upper quartiles (25-75%); whiskers extend to 10th and 90th percentiles, and all outliers are shown. **p<0.003 ***p<0.0001 and n.s.= non-significant after performed t-test analysis. Scale bar= 5µm.

3.6. ALADIN regulates the localisation of a subset of Aurora A targets

The activity of Aurora A peaks during mitosis, where it regulates a large number of spindle assembly factors (SAFs) (Barr and Gergely, 2007). Differences in the microtubules dynamic did not explain ALADIN's depletion phenotype, therefore it was decided to analyse Aurora A substrates known to be involved in spindle assembly and stabilising (Sardon et al., 2010).

ALADIN silencing produced remarkable increases in the amount of HURP and the Augmin subunit HAUS6 within the spindle (both ***$p<0.001$), and a more modest effect on Eg5/Kif11 localisation (**$p<0.05$). On the other hand, Kif2a, Tacc3, and Tpx2 were unaffected after ALADIN depletion. All Aurora A substrates tested are shown in the Figure 23. Given the results obtained, only a sub-set of Aurora A targets were altered by ALADIN depletion, suggesting that Aurora A substrates may have a different response according to the inhibition or re-localisation of Aurora A within the spindle.

HURP, which is known to be involved in k-fibres stability (Sillje et al., 2006), was spread towards the spindle pole after ALADIN-siRNA treatment, which can presumably contribute to the marked reduction of cold-stable k-fibres microtubules observed in cells lacking ALADIN.

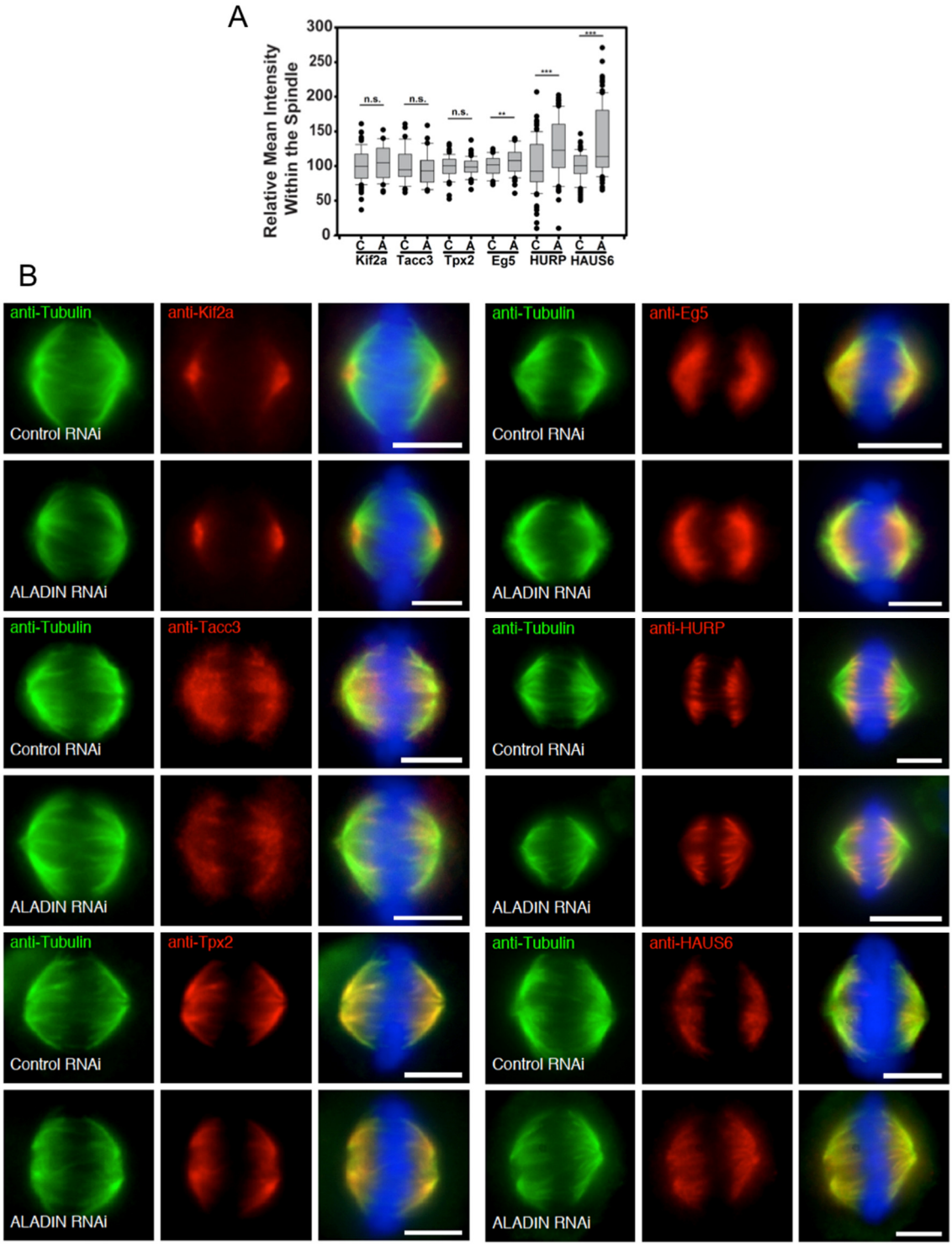


Figure 23: ALADIN silencing re-localise a subset of Aurora A substrates.
(A) The fluorescence intensity of the indicated factors within the spindle in control and ALADIN-depleted cells were measured and normalised. Only the localisation of Eg5 (0.802 *vs* 0.859), HURP (0.485 *vs* 0.607) and HAUS6 (0.680 *vs* 0.925) were affected by ALADIN depletion. All experiments were executed three times with the exception of Kif2a and HAUS6, n=2 and HURP n=4; and at least 36 cells were measured in each condition. (B) Representative Z-stacks of control or ALADIN-silenced HeLa cells arrested in metaphase for four hours and fixed and stained for the indicated antibodies. Box-and-whisker plot: middle line shows the median value; the bottom and top of the box show the lower and upper quartiles (25-75%); whiskers extend to 10th and 90th percentiles, and all outliers are shown. **p<0.05 ***p<0.0001 and n.s.= non-significant after performed t-test analysis. Scale bar= 5µm.

3.7. ALADIN is essential for NuMA localisation in an Aurora A activity-dependent manner.

The microtubule minus-end binding protein NuMA appeared to be less spread around the spindle pole and more constricted to an area around the centrosome in ALADIN depleted cells. (Figure 24A and D). Quantitative analysis of immunofluorescence images in control and ALADIN siRNA treated cells confirmed a 23% decrease in the area of spindle covered by NuMA (Figure 24B; ***$p<0.001$), and the average fluorescence intensity within the spindle was also decreased by 15% (Figure 24C; ***$p<0.001$). Western blotting analysis showed that NuMA protein levels did not change when ALADIN protein levels were reduced, suggesting a re-distribution of NuMA behind the apparent reduction in NuMA within the spindle (Figure 24E). In agreement with this idea, cortical NuMA was 22% increased in ALADIN depleted cells when compared with control conditions (Figure 24F). NuMA at the cell cortex helps to position the mitotic spindle (Kotak and Gonczy, 2013) in a complex formed with its interactor dynein (Merdes et al., 2000). After the depletion of ALADIN, the subunit p150 of the dynein activator complex was also reduced by 12.7% within the spindle (Figure 24 G; ***$p<0.001$).

It was previously reported that NuMA is an Aurora A substrate during interphase (Toughiri et al., 2013) and proposed to be a new substrate of this kinase during mitosis (Kettenbach et al., 2011; Sardon et al., 2010). Therefore, it was decided to investigate if re-localisation of NuMA in ALADIN-silenced cells was dependent upon Aurora A activity. Hence, localisation of NuMA was analysed in control and ALADIN-depleted cells with or without treatment with MLN8237. The intensity of NuMA staining within the spindle was decreased similarly by ALADIN depletion, Aurora A inhibition, or a combination of both treatments (Figure 24C and D), suggesting that NuMA's redistribution within the spindle after ALADIN depletion is Aurora A activity-dependent.

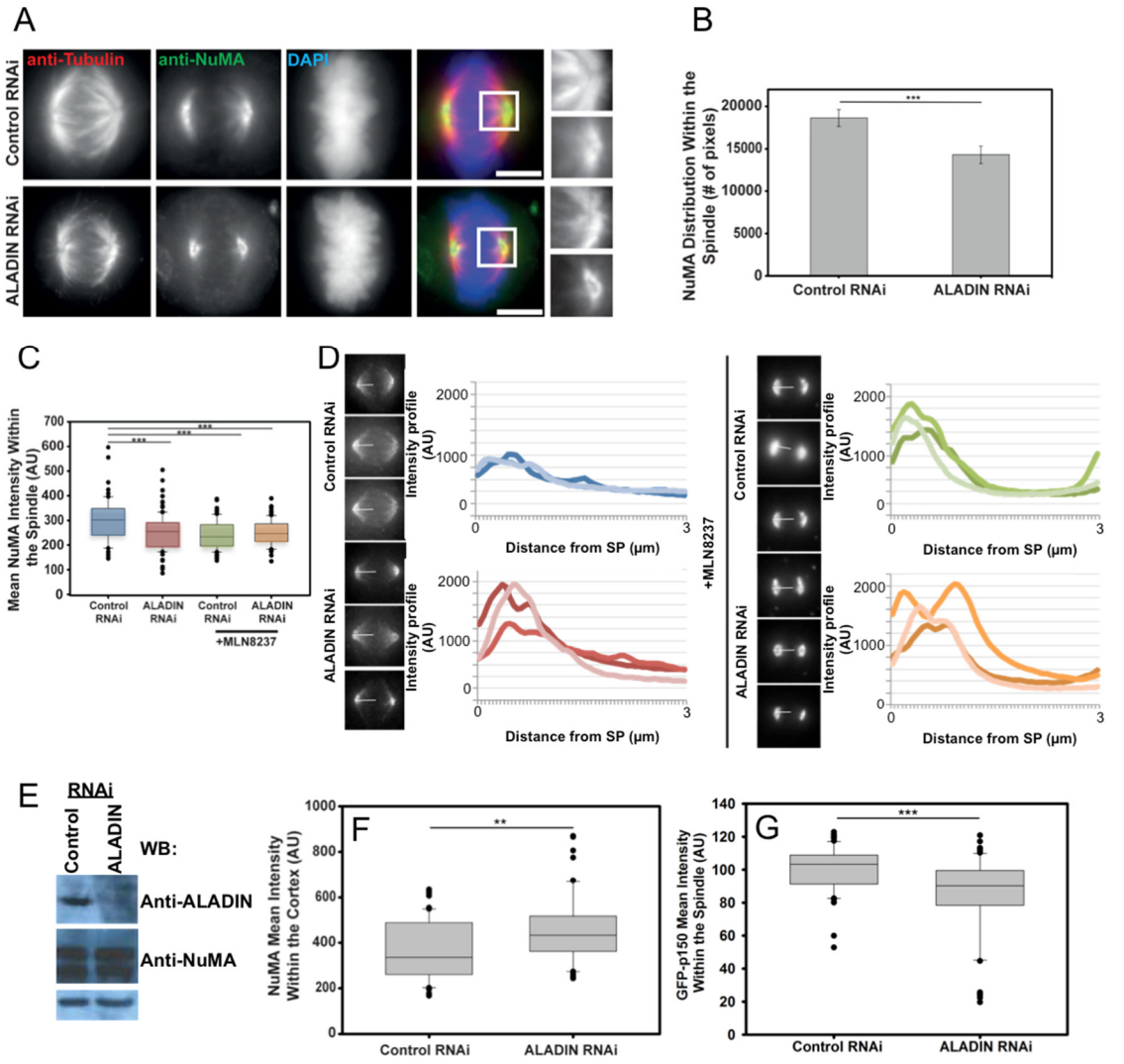


Figure 24: ALADIN is essential for NuMA localisation in an Aurora A activity-dependent manner. **(A) Representative maximum intensity projection of control and ALADIN-depleted HeLa cells fixed and stained to visualize microtubules (tubulin, red), NuMA (green), and chromosomes (DAPI, blue). For comparison purposes, magnified views of microtubules (top) and NuMa (bottom) from within the boxed regions in the merged images are presented. (B) Quantification of the number of NuMA positive pixels within the spindle is presented (18643 *vs* 14307; Mean ± SEM; at least 38 cells, n=4. (C) ALADIN depletion reduces the average NuMA intensity within the spindle in a manner similar to MLN8237, an Aurora A kinase inhibitor. Control or ALADIN-specific siRNA treated cells were incubated or not for 40min in MLN8237, then fixed and stained for NuMA and microtubules. Average of NuMA intensity was measured within the spindle of at least 33 cells from four biological replicates (mean intensities are 297.9; 253.2; 240.1; 251.8). (D) Line profile of NuMA intensity from the spindle poles (SP) in three representative cells for the indicated conditions. White line (3µm) indicates where the intensity profile line was measured. (E) ALADIN depletion does not affect NuMA protein levels. Whole cell lysates from cells treated with the indicated siRNAs were probed with anti-ALADIN, stripped and then probed with NuMA antibody. Bottom panel shows unspecific bands used as a guide of loading. See appendix for complete view of the blots used. (F) The mean intensity of NuMA on the polar cortex was measured in control and ALADIN-silenced metaphase cells. At least 50 cells were measured for each condition, n=2. (G) Box-and-whisker plot showing the average GFP–p150 intensity of cells treated with control or ALADIN siRNAs that stably express GFP-p150 from a BAC clone using an endogenous promoter. Box-and-whisker plots: Box middle line shows the median and the bottom and top lines show the lower and upper quartiles (25% and 75%). Whiskers extend between the 10th and the 90% percentile and all outliers (dots) are shown. **p<0.003 ***p<0.0001 and n.s.= non-significant after performed t-test analysis. Scale bars= 5µm.**

3.8. ALADIN is an Aurora A binding protein

Considering that ALADIN levels can influence the localisation of active Aurora A and some of its substrates, it was tested whether Aurora A could influence ALADIN localisation. For that, HeLa cells treated with MLN8237 were fixed and stained for endogenous ALADIN. In control conditions, ALADIN showed a mild enrichment around spindle poles, but after treatment with MLN8237 it became more concentrated at the spindle poles (Figure 25A). So, Aurora A activity appears to regulate ALADIN's localisation. Then, it was examined whether ALADIN could interact with Aurora A. For that, endogenous ALADIN was immunoprecipitated from cells arrested in either prophase/prometaphase (overnight treatment with STLC) or metaphase (overnight treatment with RO-3306 and then release into media with MG132 for four hours) in the presence or absence of MLN8237. In both mitotic states, Aurora A was co-precipitated only when the kinase was inhibited (+MLN8237), suggesting that ALADIN preferentially interacts with the inactive form of this kinase (Figure 25B). This protein-protein interaction was validated by the reverse immunoprecipitation of Aurora A-GFP stably expressed from a BAC clone using the endogenous promoter (Figure 25C (Poser et al., 2008)). Also, findings from the laboratory using kinase phosphorylation assay suggests that ALADIN is an *in vitro* substrate of Aurora A (data not shown, T. Kasciukovic).

So far, results have shown that active Aurora A is mis-localised from the centrosome after ALADIN depletion. While, Tpx2 is well known Aurora A substrate that also promotes Aurora A kinase activity activation within the spindle microtubules (Carmena et al., 2009), CEP192 promotes its activation at the centrosome (Joukov et al., 2010; Joukov et al., 2014). As shown before, ALADIN does not affect levels of Tpx2 within the spindle (Figure 23), hence CEP192 localisation was analysed. After treatment with ALADIN siRNA, CEP192 levels were reduced by 12% (Figure 25D), suggesting that ALADIN depletion may compromise Aurora A centrosomal localisation via CEP192. This idea is further supported by preliminary results showing CEP192 interaction with ALADIN (Figure 25E).

NEDD1 is a centrosomal protein that regulates microtubules nucleation, interacts with CEP192 and is a substrate of Aurora A (Haren, 2006; Gomez-Ferreria, 2012; Pinyol, 2013;). Quantitative analysis revealed that ALADIN depletion did not affect NEDD1 levels at the centrosome (Figure 25D). Also, pericentrin and Plk1, both centrosomal proteins, did not change their localisations after ALADIN silencing (Figure 21 and Figure 22), which together with the NEDD1 results suggests that silencing of ALADIN and the decrease of active Aurora A at the centrosome does not create substantial defects in centrosome assembly.

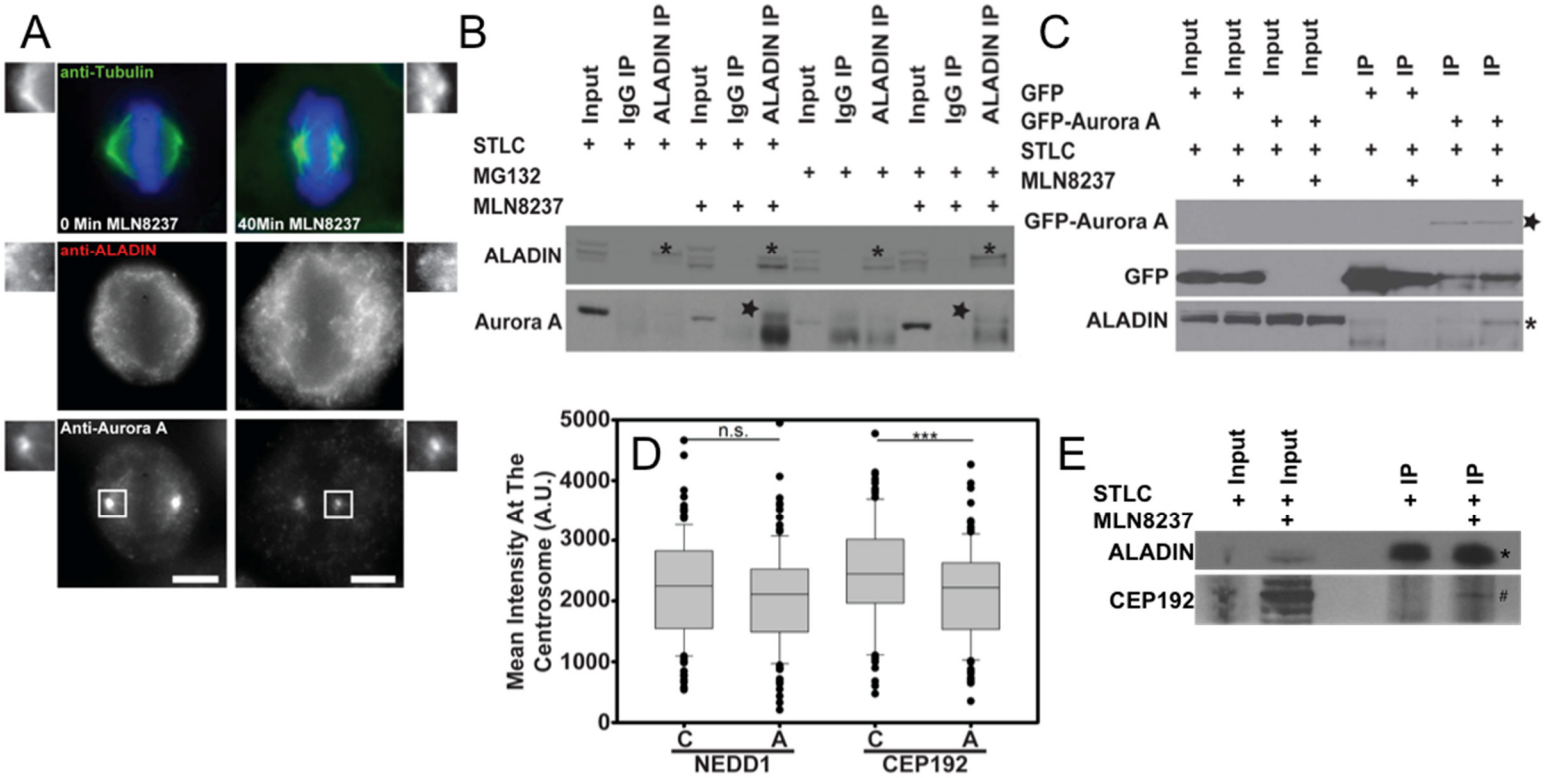


Figure 25: ALADIN binds to Aurora A and its localisation is sensitive to Aurora A activity.
(A) Representative image of HeLa cells were treated with MLN8237 to inhibit Aurora A activity. Cells were fixed and stained to visualise ALADIN, Aurora A, microtubules (Tubulin) and DNA. Magnification views of the regions marked are shown. (B) Aurora A is co-immunoprecipitated with endogenous ALADIN. Cells were arrested at either prophase/prometaphase (after an overnight treatment with STLC – STLC) or metaphase (after overnight treatment with RO-3306 and release into MG132 for four hours – MG132) and treated or not with MLN8237 prior to making lysates. 400µg of protein were used to immunoprecipitate ALADIN. Normal mouse IgG was used as a control. Immunoprecipitated complexes were analysed by western blot using the indicated antibodies. Asterisks indicate the ALADIN specific band and the star shows the Aurora A specific band. (C) Cells transiently transfected with GFP or stably expressing GFP–Aurora A were treated with STLC overnight and half were also treated with MLN8237 to inhibit Aurora A activity. 400µg of protein were used to immunoprecipitate GFP. Immunoprecipitated complexes were analysed as described in (B). Both Figures are a representative image of three independent experiments. (D) Mean intensity of centrosomal proteins NEDD1 and CEP192 were quantitated on centrosomes in cell treated with control (C) and ALADIN specific (A) siRNAs. At least 66 cells were measured, n=3. (E) Same as (B) but it was only used cells arrested in prophase/prometaphase (STLC). Hashtag indicates CEP192 band after the immunoprecipitation. ***p<0.0001 and n.s.= non-significant after performed t-test analysis. Scale bars= 5µm.

3.9.Mitotic errors are common in triple A patient fibroblasts

Triple A syndrome is caused by mutations in the gene that encodes ALADIN, and several mutations are associated with this syndrome (Krumbholz et al., 2006); hence it was asked whether these mutations could impair ALADIN's function during mitosis. To test this idea, mitotic spindles were examined in cultured fibroblasts from patients homozygously expressing the p.S263P and p.Q387X mutant forms of ALADIN (Figure 26A). Both mutant fibroblasts showed disorganised metaphase plates (Figure 26B) and shorter metaphase spindles (Figure 26C), compared to control fibroblasts and similarly with what it was found after ALADIN depletion in HeLa cells (Figure 18).

The localisation of the mutant proteins did not seem so different from the wild type ALADIN (Figure 26A and F). Next, it was examined if these proteins were recruited to the spindle pole after Aurora A inhibition as observed with wild type ALADIN in HeLa cells (Figure 25). Analogous to the wild type protein, MLN8237 treatment caused an enrichment of ALADIN S263P mutant protein. On the other hand, ALADIN Q387X mutant did not change after Aurora A inhibition (Figure 26F). Interestingly, Q387X mutant cells had a reduction in pT288 Aurora A on centrosomes although these mutant cells did not show defect in centrosomal pericentrin levels (Figure 26D). This suggests that Q387X also causes a re-localisation of Aurora A from the centrosome. In line with this idea, the distribution and amount of HURP within the spindle was significantly altered in the Q387X mutant cells. HURP spread poleward and was more concentrated on spindle microtubules (Figure 26E); implying that the reduction of active Aurora A at the centrosome in the Q387X cells had the same effect on HURP's localisation as the ALADIN depletion.

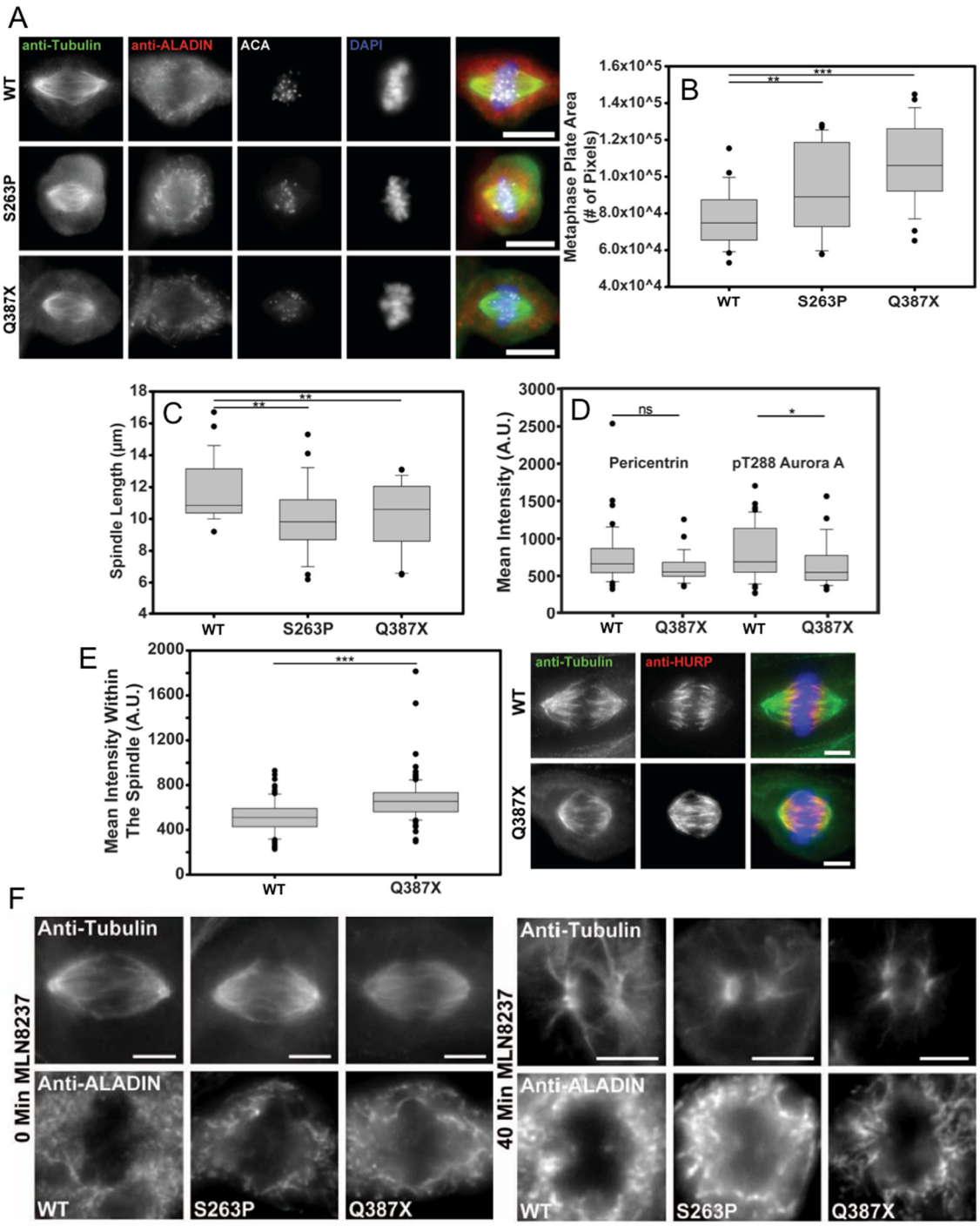


Figure 26: Triple A patient fibroblasts show errors in spindle assembly and the ALADIN disease mutants localise differently than the wild-type protein.
(A) Control fibroblasts and fibroblasts from patients expressing two different disease mutant forms of ALADIN, p.S263P and p.Q387X were fixed and stained to visualise the spindles. The metaphase plate area (B) (n = 24 for WT, n = 26 for S263P, and n = 27 for Q387X) and spindle length (C) (n = 26 for WT, n = 27 for S263P, and n = 25 for Q387X) were measured for conditions. (D) Mean intensity of active Aurora A at the centrosome in the indicated fibroblasts. Phospho-T288 intensity was measured and restricted to the centrosomal region using Pericentrin labeling to define the centrosome. Pericentrin levels are not compromise in Q387X ALADIN fibroblasts. 42 WT and 29 Q387X fibroblasts from two independent experiments were measured. (E) Quantification of HURP intensities within the mitotic spindle (left panel; at least 58 cells were analysed in each condition). A representative immunofluorescence image of the cells used for this quantification is shown on the right panel. (F) Fibroblasts expressing the given forms of ALADIN were treated with MLN8237 for 40 minutes to inhibit Aurora A, fixed, and then stained to visualise microtubules (tubulin) and

ALADIN. Box-and-whisker plot: middle line shows the median value; the bottom and top of the box show the lower and upper quartiles (25-75%); whiskers extend to 10th and 90th percentiles, and all outliers are shown. *p<0.1, **p<0.05, ***p<0.001 and n.s= non-significant after performed t-test analysis. Scale bar = 5 µm.

3.10.Discussion

ALADIN has previously been studied in the context of the function of adrenal and neuronal cells in triple A syndrome, and many of the phenotypic features seen in triple A patients have been associated with this protein's role in nucleocytoplasmic transport (Juhlen et al., 2015; Prasad et al., 2013; Sarathi and Shah, 2010). Here it was shown for the first time that this protein also has functions in cell division. Without ALADIN, slower spindle assembly and chromosome alignment were observed. Furthermore, ALADIN is required for regulating spindle length and the formation of stable k-fibre microtubule bundles.

The activation and localisation of Aurora A has previously been shown to be controlled by many specific protein cofactors (Bayliss et al., 2003; Hirota et al., 2003; Hutterer et al., 2006; Joukov et al., 2010; Joukov et al., 2014; Kufer et al., 2002; Reboutier et al., 2012; Satinover et al., 2004; Zhao et al., 2005; Zorba et al., 2014). Here it was demonstrated that ALADIN is a novel Aurora A cofactor that promotes the localisation of the active form of this kinase at centrosomes.

Aurora A dynamically associates with the centrosome and spindle poles (Lioutas and Vernos, 2014), and here it was described that both reducing and increasing ALADIN protein levels leads to a diminished amount of active centrosomal Aurora A. Also, ALADIN preferentially interacts with inactive Aurora A and evidences from the laboratory suggests that ALADIN is a new substrate of Aurora A. Given these data, it is hypothesised that ALADIN could act to locally anchor inactive Aurora A that is released from spindle poles and promote its reactivation by other cofactors. For instance, when Aurora A is phosphorylated (normal conditions) ALADIN is kept away from the spindle pole. On the other hand, if Aurora A is inactive (after treatment with MLN8237), ALADIN is free to move towards the spindle pole. However, an overabundance of ALADIN could disrupt this dynamic and outcompete other cofactors and prevent the subsequent reactivation and recruitment of the active kinase to the centrosome. To test this hypothesis, it was started a collaboration with Dr. Richard Bayliss Lab from the University of Leicester to assess the effect of ALADIN on Aurora A kinase activation and activity *in vitro*. Unfortunately, this assay it was not possible due to an inability to produce ALADIN protein.

CEP192 is required to the recruitment and activation of most Aurora A at the centrosome (Joukov et al., 2014). Results here presented suggest that ALADIN interacts with CEP192 after MLN8237 treatment, and that ALADIN depletion reduces the amount of CEP192 at the centrosome. Therefore, Aurora A recruitment and activation at the centrosome could be dependent on a complex formed by CEP192, ALADIN and inactive Aurora A. Further studies are necessary to confirm the presence of this complex and better clarify the mechanism that underlie the spatial control of Aurora A by ALADIN.

A spindle matrix built of components including nucleoporins, lamins and vesicles from Golgi and ER has been proposed to function as a non-microtubule scaffold that can concentrate and/or activate spindle assembly regulators (Schweizer et al., 2014; Zheng, 2010). Although it is not possible to say anything definitive about the existence or function of the spindle matrix, ALADIN's localisation and role in regulating Aurora A function are both consistent with the proposed nature of a matrix component.

It was also shown that ALADIN silencing specifically affects a subset of Aurora A substrates (NuMA, HURP, and Augmin) but not others (Plk1, Kif2a, Tacc3, Tpx2, NEDD1). Since ALADIN regulates only Aurora A localisation but not the overall protein amount, these findings demonstrates that there are multiple and separable pathways by which Aurora A can regulate its substrates and reinforces the idea that the spatial regulation of this kinase is essential for its proper function at spindle assembly.

While ALADIN's depletion perturbs the localisation of NuMA and generates spindle poles that are less focused, a total loss of NuMA abalates spindle focusing (Silk et al., 2009), suggesting that there is still NuMA function in the absence of ALADIN. Since the NuMA localisation phenotype is similar in cells lacking ALADIN or when Aurora A activity is inhibited (+MLN8237), ALADIN depletion may leads to a hypo-phosphorylation of NuMA but does not completely eliminate its function in spindle focusing.

In addition to NuMA localisation, ALADIN was shown to regulate the localisation of HURP and the augmin complex. It has been described that HURP localisation is controlled by both Ran-GTP

dependent release from importin beta and Aurora A phosphorylation (Sillje et al., 2006; Wong et al., 2008). Given that no defects in Tpx2 localisation were observed in ALADIN-depleted cells, it can be assumed that Ran gradient is not severely perturbed in these cells; and the poleward spread of HURP observed may be due to the re-distribution of Aurora A.

The regulation of Augmin protein complex by Aurora A is not as clear as for HURP. Phosphorylation by this kinase on the Hice1 component affects microtubule nucleation (Tsai et al., 2011). Furthermore, depletion of the Augmin complex components in HeLa cells triggers the spindle checkpoint and reduces tension on sister kinetochores (Uehara et al., 2009). Although no compensation or overexpression studies have been made to understand the impact of HAUS6 within the spindle, it can be discard the possibility that increased kinetochore tension observed after ALADIN depletion is caused by des-regulation of the Augmin complex. Nevertheless, HURP can also contribute to kinetochore stretch as HURP associates with the spindle and stabilises the k-fibre microtubules (Sillje et al., 2006; Ye et al., 2011). Increased kinetochore stretch and destabilised k-fibres within the spindle are a phenotype somehow unusual. Although both are related, attachment stability and k-fibre numbers are two separate things (King and Nicklas, 2000). Nevertheless, Meunier and colleagues reported that depletion of MCRS1, which caps microtubule minus ends, produced a very similar phenotype of shorter, destabilised spindles with hyper-stretched kinetochores (Meunier and Vernos, 2011). Additionally, unlike what it was observed for ALADIN, MCRS1 regulates microtubule flux during metaphase (Meunier and Vernos, 2011); suggesting that there are multiple ways in which tension within the spindle may be dis-regulated.

Taken together, all these data suggest that ALADIN depletion spatially alters Aurora A activity to selectively change the distribution of a specific suite of spindle assembly and stabilising factors within the spindle. So, it is hypothesised that when ALADIN is removed, the resulting change in the spatial localisation of active-inactive form of Aurora A, through their substrates, produces the increased kinetochore stretch, destabilised k-fibres, and delayed chromosome alignment observed.

During normal cell cycles, none of the defects seen after ALADIN depletion are enough to block or interrupt cell divisions, as mice without ALADIN are viable (Huebner, 2006). Once ALADIN-depleted cells complete chromosome alignment, they progress to anaphase without lagging chromosomes or other hallmarks of mitotic errors; implying that in most cells, ALADIN's role in regulating Aurora A function makes spindle assembly more robust. However, different cell type exhibits different mitotic dynamics and there is the possibility that this role could become more critical in different tissue types.

While it is well described that mutations in ALADIN lead to triple A syndrome, no genotype-phenotype correlation has been identified (Huebner et al., 2000; Sarathi and Shah, 2010). It has been proposed that alterations in reactive oxygen levels, steroidogenesis or defects in nucleotide excision repair produce some symptoms seen in patients who suffer from triple A syndrome (Juhlen et al., 2015; Kind, 2010; Kind et al., 2009; Kiriyama et al., 2008). Here it is shown for the first time that the expression of two mutant forms of ALADIN in patient cell lines produces specific defects in mitotic spindle formation. In particular, Q387X ALADIN mutant causes a reduction of active Aurora A at the centrosome. Interestingly, this mutant protein is truncated on their C-terminus and contrarily to the wild type and S263P mutant protein, does not respond to the MLN8237 treatment. Hence, it could be hypothesised that the C-terminus of ALADIN is crucial for ALADIN's dynamics within the spindle pole involved in the regulation of Aurora A recruitment to the centrosome. Thus, further work will be needed to understand this relation and determine to what extent mitotic spindle assembly errors contribute to the etiology of triple A syndrome either alone or in combination with deficits in DNA repair or reactive oxygen/superoxide homeostasis that have already been documented in patient cells.

4.Characterisation of ALADIN's function during meiosis

The work presented in this chapter was done in collaboration with Professor Angela Huebner (Children's Hospital) and Professor Rolf Jessberger (Institute of Physiological Chemistry, Medical Faculty Carl Gustav Carus) at the University of Technology, Dresden, Germany.

4.1.Introduction

Accurate chromosome segregation during mitotic and meiotic cell division is largely dependent on the proper assembly and orientation of the microtubule spindle (McNally, 2013; Ohkura, 2015). Spindle orientation determines where division takes place, and consequently the size of each daughter cell (McNally, 2013). In the large majority of animal cells the spindle is located at the centre of the cell, and after division two daughter cells of equal size are generated; polarised cells have asymmetrically positioned spindles. Mouse oocytes have extremely asymmetrically positioned spindles (Brunet and Maro, 2007; Fabritius et al., 2011). During meiosis I, after the spindle assembles in the oocyte centre, it migrates towards the nearest cortex. Once that one of the spindle pole reaches the cortex, the spindle rotates and adopts a perpendicular orientation relative to the cortex (Fabritius et al., 2011; McNally, 2013). At anaphase onset, the spindle is stably anchored to the cortex, and chromosomes are extruded into small, non-developing polar bodies (Fabritius et al., 2011; McNally, 2013). This asymmetric division preserves almost all the cytoplasmic content within the oocyte, which is essential for fertilisation (Chaigne et al., 2012; Fabritius et al., 2011).

Regulation of meiotic spindle positioning is dependent upon actin, myosin II and formin (Dumont et al., 2007a; Schuh and Ellenberg, 2008; Verlhac et al., 2000; Yi and Li, 2012). But, very little is known about the mechanisms that govern spindle assemble and positioning during oocyte maturation (Brunet and Maro, 2007; Chaigne et al., 2012; Fabritius et al., 2011; Howe and FitzHarris, 2013; McNally, 2013). Infertility can be genetically caused by defective meiotic divisions (Ohkura, 2015). Homozygous ALADIN knockout mice are viable; however, females are sterile (Huebner, 2006), suggesting that ALADIN could be required for oocyte maturation and production of functional female gametes.

ALADIN was first discovered as a protein of the nuclear pore complex that participates in the control of nuclear transport during interphase (Huebner et al., 2000). Mitotic studies in human cells have now uncovered ALADIN's role in proper regulation on the spindle assembling via the spatial regulation of Aurora A kinase (Carvalhal et al., 2015). Depletion of ALADIN in human

cells slows the spindle assembling and causes shorter bipolar mitotic spindles (Carvalhal et al., 2015).

Here it is reported ALADIN involvement in meiotic spindle positioning and spindle assembly during meiosis I in mouse oocytes. ALADIN's deletion disturbs oocyte maturation leading to unfertile eggs.

4.2.Mitotic ALADIN's localisation is conserved during mouse oocyte meiotic division

During interphase, ALADIN is localised at the nuclear pore complex (Cronshaw et al., 2002). As described in Chapter 3 in mitotic human cells, ALADIN localises around the mitotic spindle and at spindle poles. To test whether ALADIN's localisation is conserved during meiotic divisions, wild type mouse oocytes at metaphase I and II were fixed and stained with an ALADIN specific antibody. During metaphase I, ALADIN mainly accumulated around the meiotic spindle (Figure 27A) and its localisation was maintained throughout metaphase II (Figure 27B). Additionally, ALADIN appeared to remain partially associated with the spindle during its migration to the cortex. To confirm ALADIN's localisation, oocytes were injected with capped mRNAs encoding GFP-ALADIN and H2B-RFP and imaged until metaphase I. Before NEBD, GFP-ALADIN was localised around the germinal vesicle (GV; nucleus, Figure 27C). When chromosomes were fully condensed and aligned at the metaphase plate, ALADIN was localised around the metaphase plate, where the spindle is expected (Figure 27C). Interestingly, GFP-ALADIN was also observed in clusters across the cytoplasm, potentially caused by the overexpression of this protein.

Overall, these results suggest a conservation of ALADIN's localisation around the spindle in somatic and germ cells.

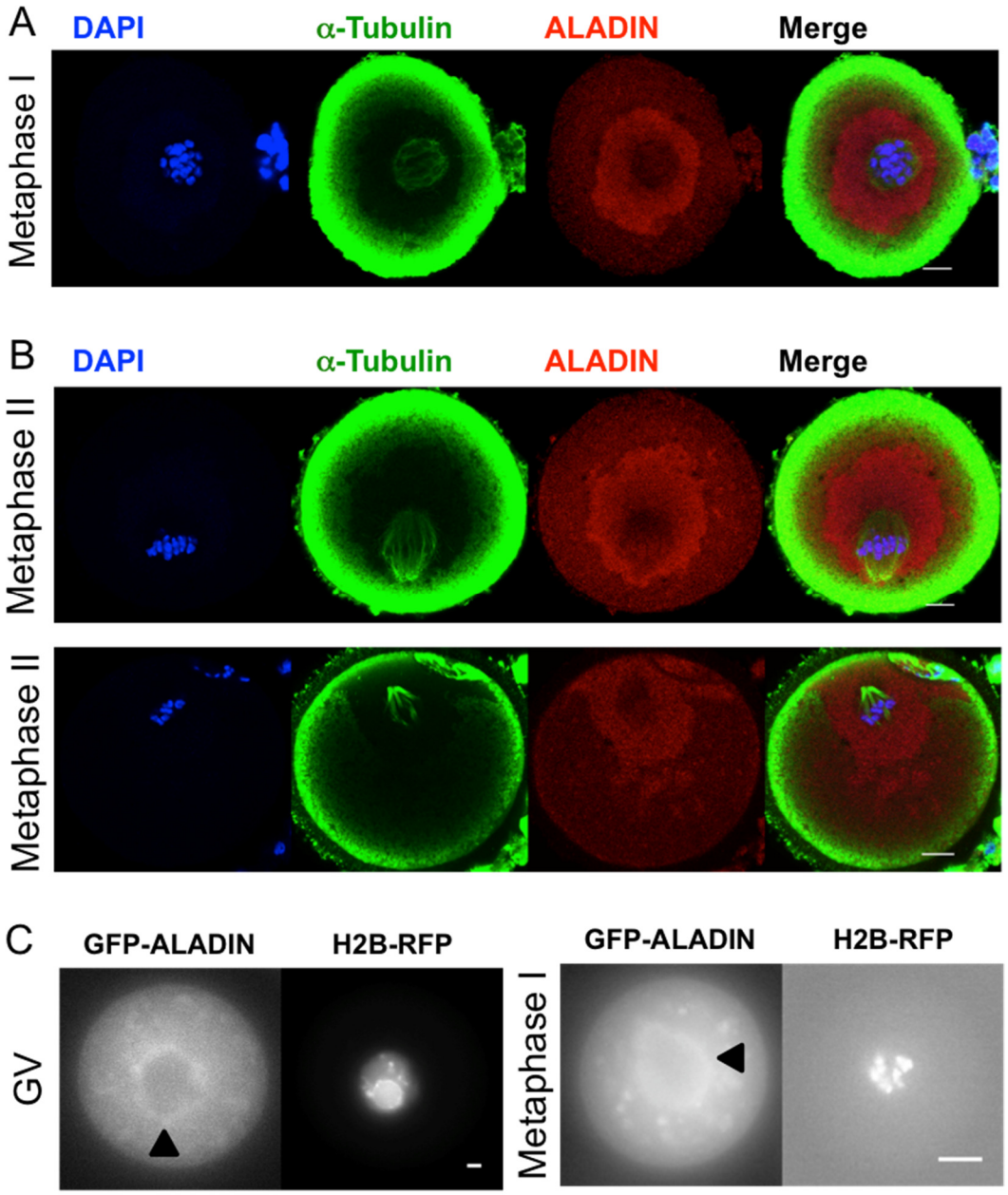
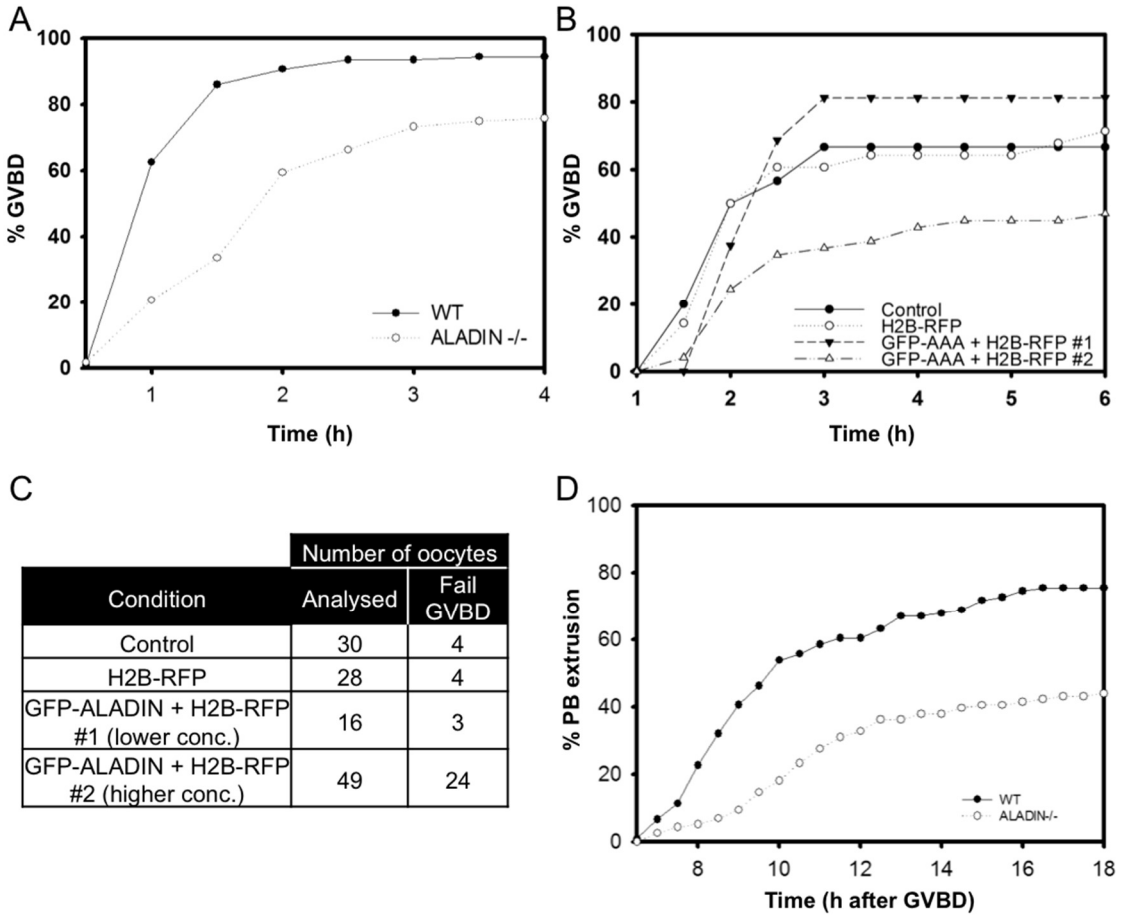


Figure 27: ALADIN's localisation during mouse meiotic oocyte maturation.
Representative Z-stack images of fixed and stained oocytes at metaphase I (A) and II (B) for the indicated antibodies. (C) Representative Z-stack images of ALADIN's localisation in live oocytes expressing GFP–ALADIN and H2B–RFP at germinal vesicle (GV) stage (left panel) and at metaphase I (right panel). Scale bars= 20 µm.

4.3.Deletion of ALADIN disturbs oocyte maturation *in vitro*

ALADIN female homozygous knockout mice (ALADIN $^{-/-}$) were found to be viable but sterile (Huebner, 2006), suggesting that ALADIN could impair oocyte maturation to produce non-functional female gametes. Therefore, it was decided to analyse the meiotic progression of ALADIN $^{-/-}$ oocytes and wild type oocytes (WT; ALADIN $^{+/+}$). Oocytes were collected in a media containing milirone to preserve an intact GV by prophase arrest. Two hours after resumption of meiosis, almost all WT oocytes had undergone germinal vesicle breakdown (GVBD); however, ALADIN KO oocytes required significantly longer to show GVBD (Figure 28A; 1.9 h ± 1.3 *vs* 2.7 h ± 1.6; ***p<0.001). Strikingly, when oocytes were injected with high levels of ALADIN mRNA, 29 of the 49 WT oocytes analysed did not undergo GVBD. This phenotype was rescued by injecting less of the ALADIN mRNA; suggesting that ALADIN overexpression can have a dominant negative effect in meiosis like in mitosis (Figure 28B and C).

Most of WT oocytes extruded their first polar body (PB) ten hours after GVBD. In contrast, only 50% ALADIN$^{-/-}$ oocytes extruded their polar body (Figure 28D). This process was also significantly slower in the ALADIN KO than the WT (Figure 28D; WT: 9.6 h ± 2.9 *vs* ALADIN$^{-/-}$:11 h ± 2.5; **p<0.007).



Condition	Number of oocytes	
	Analysed	Fail GVBD
Control	30	4
H2B-RFP	28	4
GFP-ALADIN + H2B-RFP #1 (lower conc.)	16	3
GFP-ALADIN + H2B-RFP #2 (higher conc.)	49	24

Figure 28: ALADIN is essential for proper GVBD timing and polar body extrusion.
(A) Cumulative percentage of GVBD along the time (h, hours) in WT and ALADIN $^{-/-}$ oocytes. More than 100 oocytes were measured from 5 independent experiments. Average GVBD time in WT: 1.9 h ± 1.3 and ALADIN $^{-/-}$: 2.7 h ± 1.6, ***p<0.001. (B) WT oocytes were injected with the indicated mRNAs and cumulative percentage of GVBD was measured along the time. As control no injected oocytes were used and similar amount of mRNA were added to the injection needle in all remaining conditions, but ALADIN mRNA concentration was twice concentrated in #2 (8 µg/µL) than #1 (4.4 µg/µL). (C) Absolute number of oocytes analysed in (B) and how many of them remained arrested in GV during all the time course of the experiment (6 h; fail GVBD). Mean ± standard deviation for Control: 2.154 ± 0.5; H2B-RFP: 2.438 ± 1.1; GFP-ALADIN + H2B-RFP #1: 2.346 ± 0.4 and GFP-ALADIN + H2B-RFP #2: 2.62 ± 1.0h. All experiments were performed at least 3 times with the exception of GFP-ALADIN + H2B-RFP #1, n=1. (D) Percentage of first polar body extrusion over time in WT *vs* ALADIN$^{-/-}$ oocytes. n=5, more than 100 oocytes quantified. Mean ± standard deviation 9.6 h ± 2.9 *vs* 11 h ± 2.5; **p<0.007.

4.4. ALADIN assists correct spindle positioning in oocyte asymmetric cell division

So far it was shown that the ability of oocytes to undergo GVBD and extrude PB is compromised in the absence of ALADIN. As described in the Chapter 3, a lack of ALADIN in mitosis of somatic cells impairs spindle assembly and produces shorter spindles. As ALADIN's localisation is conserved around the spindle in both cell models, it was tested if ALADIN could also participate in spindle assembly during oocyte cell division. Thus, meiotic spindle assembly at metaphase I with and without ALADIN was analysed by time-lapse using fluorescent tubulin (GFP-β-Tubulin) and histone (H2B-RFP) as spindle markers. Representative frames of this time course are shown in Figure 29. After injection, ALADIN$^{-/-}$ oocytes, as observed previously, took longer to achieve GVBD and had a reduced ability to extrude polar bodies (Figure 30A and B). This similar timing suggested that the injection and increased tubulin expression was not contributing to the ALADIN$^{-/-}$ phenotype.

On average, ALADIN$^{-/-}$ oocytes took 126 minutes more to assemble a bipolar spindle after GVBD than wild type oocytes (Figure 30C; 3.0 h ± 1.9 *vs* 4.6 h ± 3.2; **p<0.05). Also, these bipolar structures were significantly shorter when compared with WT spindles (Figure 30E; **p<0.05).

Asymmetrically positioned meiotic spindles expel chromosomes into minuscule, non-developing polar bodies (Fabritius et al., 2011; McNally, 2013). Thus, to visualise spindle relocation to the oocyte cortex, the time from spindle bipolarisation to when it contacts the oocyte cortex was measured (Figure 30F). ALADIN null oocytes showed a slower, but not significantly, migration of the spindle to the cortex, when compared with WT oocytes (Figure 30 F; 2.8h ± 1.2 *vs* 3.5 h ± 2.7).

Polar body extrusion is mediated by the meiotic spindle, which is attached to the oocyte cortex by one pole. Therefore, the angle formed between this spindle pole and the cell periphery was evaluated (Θ, Figure 30D), in oocytes able to extruded their polar body (referred as PB) or not

(NO PB). This angle was measured when the spindle first reaches to the oocyte cortex. In wild type conditions, independent of their ability to extrude polar bodies (PB and NO PB), spindles interacted with the oocyte cortex at similar angle (Figure 30G). However in the case of ALADIN$^{-/-}$ conditions, only oocytes that could extrude polar bodies (PB; Figure 30G) interacted with the cortex at the stereotypical angle. These results suggest that ALADIN null oocytes fail in polar body extrusion due to problems in the asymmetric positioning of the meiotic spindle relative to the oocyte cortex.

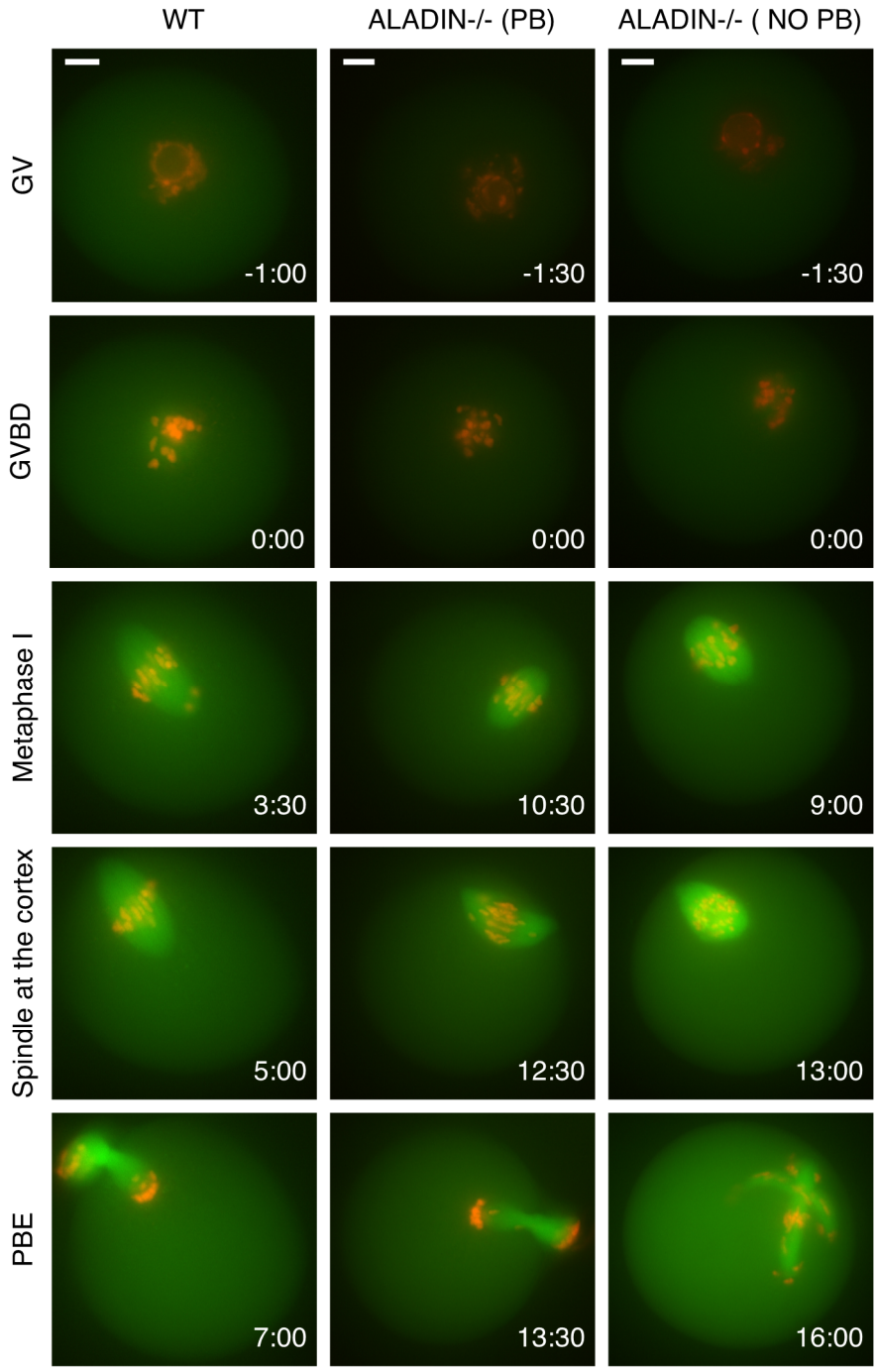


Figure 29: Oocytes lacking ALADIN show impaired spindle positioning and have less robust spindles. Representative images of oocytes injected with capped mRNA GFP-β-Tubulin and H2B-RFP during oocyte maturation. Time 0:00 represents GVBD. GV – Germinal Vesicle; GVBD – Germinal Vesicle Breakdown and PBE – Polar body extrusion. Scale bars= 10 µm.

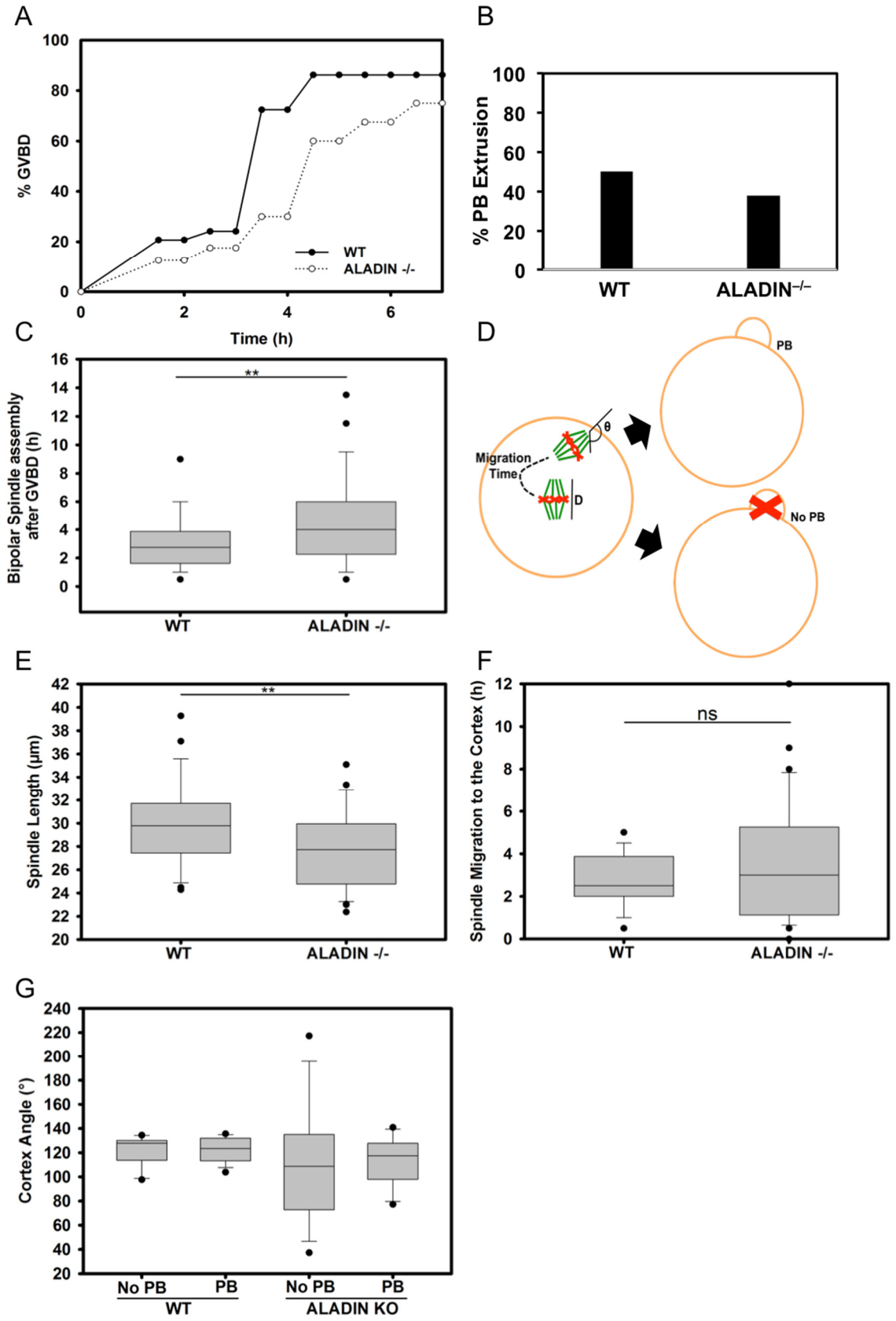


Figure 30: ALADIN is required for correct spindle assembly and positioning in oocyte asymmetric cell divisions.
(A) Cumulative percentage of GVBD along the time (h, hours) in WT and ALADIN $^{-/-}$ oocytes expressing GFP-β-Tubulin and H2B-RFP. (B) Percentage of polar body extrusion on the conditions described in (A). 50% *vs* 38.0%. (C) Setup of spindle bipolarity is delayed when ALADIN is lost (3.0

h ± 1.9 *vs* 4.6 h ± 3.2; **p<0.005). Bipolarity assembly was scored when two poles were distinguishable and normalised to the GVBD time. (D) Explanatory diagram of the quantifications made on (E-G) figures. Where D represents the pole-pole distance within the spindle at metaphase I. Metaphase I time was defined when bipolar spindles structures have the most compact and organised metaphase plate. The grey dotted line represents the migration time from the spindle in metaphase I (at the centre) to the oocyte cortex. Θ represents the angle made by the vectors spindle pole-cortex and spindle pole-bipolar structure. (E) Box-plot showing the spindle length (D) of oocytes WT and ALADIN$^{-/-}$ expressing GFP-β-Tubulin and H2B-RFP. ALADIN$^{-/-}$ oocytes have a significant short spindle in metaphase, 29.9 µm ± 3.6 *vs* 27.5 µm ± 3.3, **p<0.05. (F) The migration time of the spindle as explain in (D) was measured for the indicated conditions (2.8h ± 1.2 *vs* 3.5 h ± 2.7). ns= no significant. (G) The cortex angle represented and explained in (D) as Θ was measured and sub-divided into the following categories: PB – Oocytes that have extruded their polar body and No PB – Oocyte that after have their spindle at the cortex have fail to extrude their polar body. WT PB: 122.3º ± 11.8, WT NO PB: 122.5º ± 9.6, ALADIN$^{-/-}$ PB: 113.1º ± 19.9 and ALADIN$^{-/-}$ NO PB: 110.3º ± 48.4. All figures represent the results obtained by the analysis of four independent experiments executed with similar concentrations of GFP-β-Tubulin and H2B-RFP mRNAs (WT= 26 and ALADIN$^{-/-}$= 36 oocytes).

4.5.ALADIN is essential for fertile egg production and female fertility in mice.

So far, it was shown that ALADIN$^{-/-}$ oocytes have problems at GVBD timing, spindle assembly, asymmetric spindle positioning and relocation, and polar body extrusion. However, only around 50% of ALADIN$^{-/-}$ oocytes have shown these impairments, while the other 50% of ALADIN$^{-/-}$ oocytes showed a normal phenotype when compared with WT. Therefore, it was reasoned that ALADIN$^{-/-}$ female mice sterility could be driven by abnormal chromosome segregation from the diploid to haploid state. Thus, chromosome number and structure was analysed only on the group of ALADIN$^{-/-}$ oocytes that shown a normal phenotype (metaphase II arrested oocytes with extruded polar bodies). Surprisingly, chromosome spreads for both WT and ALADIN$^{-/-}$ appeared very similar and showed no differences in chromosome pairing (Figure 31A). Given these results, it was hypothesised that the female sterility problems could be caused by meiotic divisions errors, which could give rise to aneuploid embryos upon fertilisation. To test this idea, laser-assisted *in vitro* fertilisation (IVF) was done using oocytes isolated from their follicles and fully matured *in vitro* (eggs arrested at metaphase II) and WT (ALADIN$^{+/+}$) spermatozoa. Laser-assistance facilitates fertilisation by eliminating problems of spermatozoa penetration through the *zona pellucida* (Woods et al., 2014). A total of 27 fully matured oocytes from WT and 15 from ALADIN$^{-/-}$ mice were successfully lasered (these oocytes where collected from 3 adult mice in each condition).

On the following day, almost a third of WT eggs effectively generated two-cell embryos (9 of 27), confirming the success of IVF. On the other hand, only one of 15 ALADIN$^{-/-}$ eggs generated a two-cell embryo (1 of 15; Figure 30B). From the fertilised ALADIN$^{-/-}$ eggs, three clear abnormal two-cell embryos were observed, which did not develop further and were dead at the end of the procedure (see asterisk, right column on Figure 32). All the WT embryos developed successfully until the final possible *in vitro* stage for embryos (blastocyst stage). On the contrary, the single two-cell embryo found in ALADIN$^{-/-}$ conditions remained arrested until the end of this experiment (middle column on Figure 32).

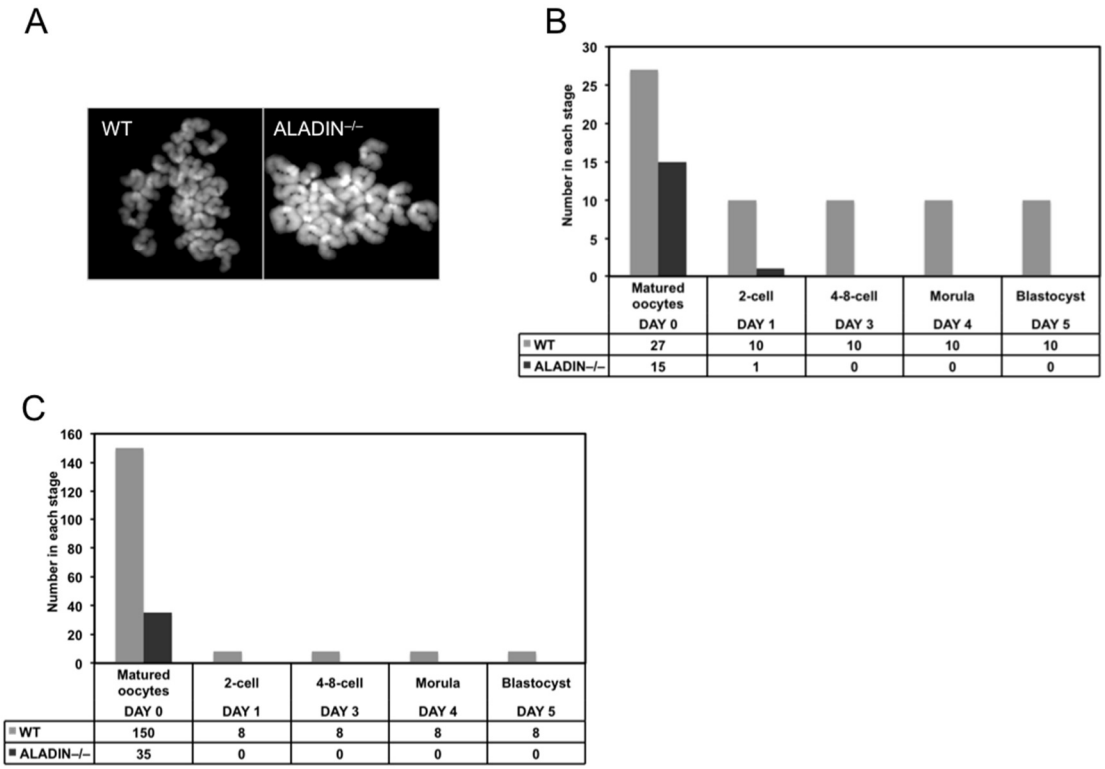


Figure 31: ALADIN$^{-/-}$ is required for to two-cell stage of mouse development female.
(A) Representative chromosome spreads of WT and ALADIN$^{-/-}$ oocytes arrested at metaphase II and with PB presented. (B) Quantification of cell embryo stages during mouse development stages until blastocyst of WT and ALADIN$^{-/-}$ oocytes fertilised with WT spermatozoa using laser assisted IVF. Three adult female mice were sacrifice in each condition and their oocytes collected. Occytes were then incubated in M2 medium for 14 h to fully mature. Only oocytes with PB presented were used for IVF. (C) Same as before, but using conventional IVF and oocytes from superovulated mice. None of the ALADIN$^{-/-}$ egg could successfully be fertilised.

Given that oocytes are very sensitive cells, this procedure was repeated using eggs obtained via superovulation and conventional *in vitro* fertilisation. Superovulation preserves the natural environment over the entire course of meiotic maturation of the oocytes, discarding problems with the isolation and maturation of oocytes associated with the previous procedure. Using conventional *in vitro* fertilisation, the ALADIN$^{-/-}$ group completely failed to generate symmetric two-cell embryos (Figure 31 and Figure 33) when compared to the WT group, which successfully develop until the blastocyst stage.

These results suggest that ALADIN expression is crucial for proper mouse oocyte maturation and is required for fertilisation and early embryo development in mice.

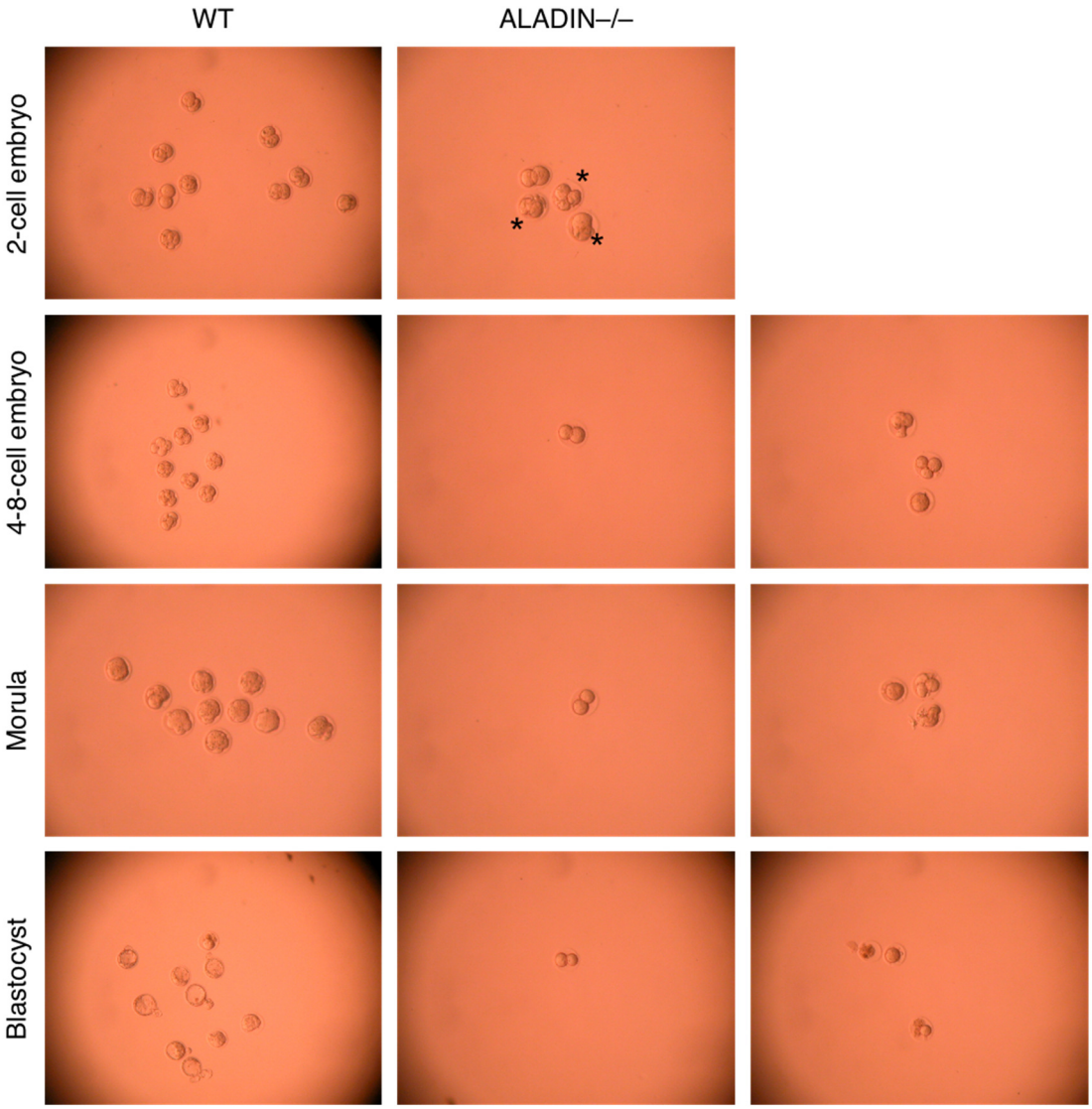


Figure 32: Cell-embryo mouse development of WT and ALADIN$^{-/-}$ oocytes matured *in vitro* and WT spermatozoa using laser assisted IVF.
Asterisks indicate cell-embryos with abnormal division. This group was then separated, column on the right side. They were dead on the blastocyst stage, 5 days after fertilisation.

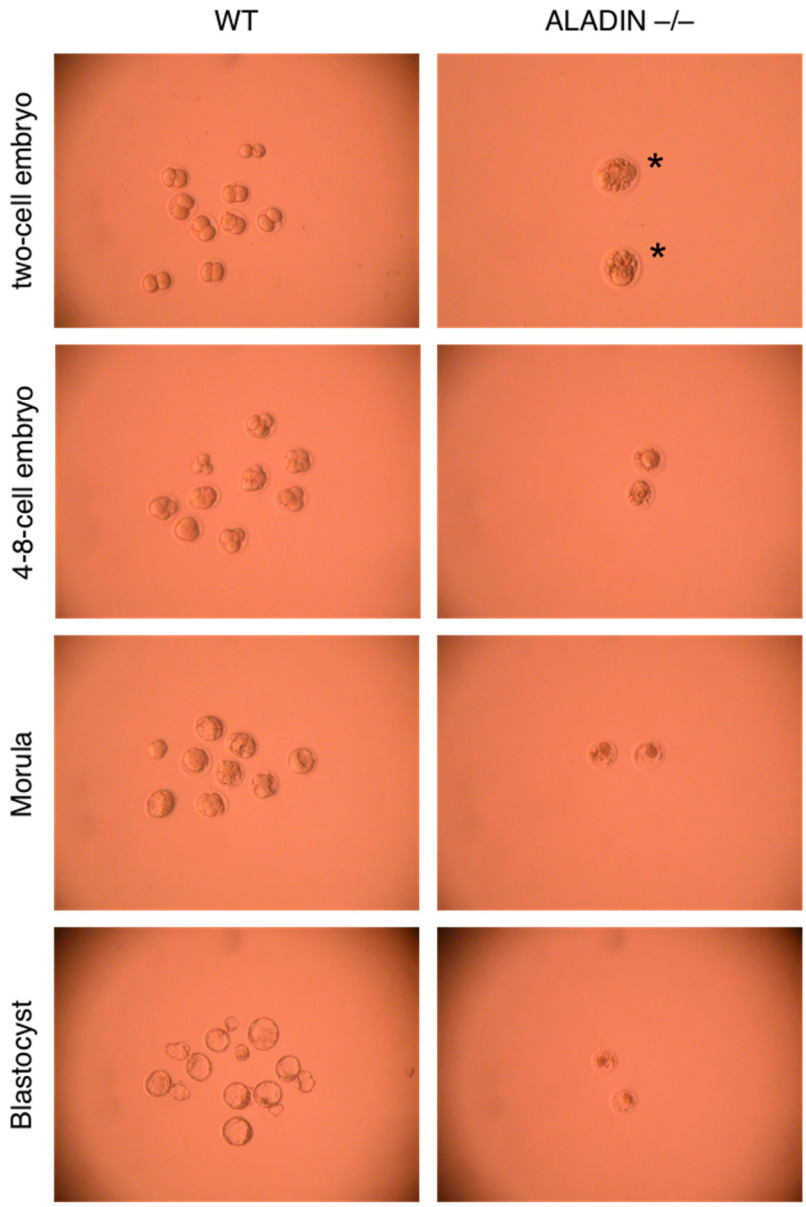


Figure 33: Cell-embryo mouse development of WT and ALADIN$^{-/-}$ oocytes matured using super-ovulation and WT spermatozoa.
After conventional IVF, 2-cell embryos were isolated and pictures took during the next five days (blastocyst stage). Asterisks indicate cell-embryos with abnormal division.

4.6.Discussion

Nucleoporins (NUPs) are components of the nuclear pore complex (NPC), which is essential for nuclear transport during interphase, but several nucleoporins are also known to be involved in chromosome segregation during mitosis. On the previous chapter the NUP ALADIN has been identified with functions in mitotic spindle assembly using human cells. Here it was shown for the first time that ALADIN also has a role during meiosis, and is essential for mouse oocyte maturation and embryo development. These data demonstrate that similarly with what happens in mitosis in mammalian cells (Chatel and Fahrenkrog, 2011), NUPs also have a role in mouse female meiosis.

After long periods in prophase I arrest, meiotic resumption is initiated by the breakdown of the nuclear envelope of the germinal vesicle (GV) triggered by the Maturation Promoting Factor (MPF) (Howe and FitzHarris, 2013). MPF enters the nucleus and phosphorylates NUPs promoting NPC disassembly (Margalit et al., 2005). Here it has been shown that levels of ALADIN are important for GV breakdown (GVBD) timing and progression. In fact, overabundant ALADIN causes almost 50% of oocytes to arrest at the GV state. Although previous studies in mammalian cells demonstrated that ALADIN is not necessary for nuclear envelope breakdown (NEBD), disassemble or reassembly (Kind et al., 2009; Krumbholz et al., 2006); its interactor NCD1 (Kind et al., 2009) is required for NPC assembly in vertebrate cells (Haren et al., 2006). Therefore, it cannot be exclude the possibility that ALADIN affect GVBD via its interactor protein NCD1.

Lack of ALADIN not only affects GVBD, but also slows spindle formation, migration and reduces oocytes ability to extrude PB. Thus, ALADIN is a novel key factor for oocyte meiotic division. Depletion of ALADIN from mammalian cells slows chromosome alignment, and assembled spindles are shorter than control conditions. Once ALADIN depleted cells progress to anaphase, chromosomes are properly segregated, showing that while ALADIN is dispensable for mitosis, it helps in the assembly of a more robust spindle. In oocyte meiosis, similar results were obtained. Bipolar spindle formation was delayed in ALADIN$^{-/-}$ oocytes when compared with wild

type oocytes, and meiotic spindles at metaphase I are shorter. As in mitosis, chromosome spreads of ALADIN$^{-/-}$ oocytes show normal chromosome segregation. Therefore, ALADIN contributes to the robustness of the spindle in mitosis and meiosis.

Oocytes divide extremely asymmetrically to preserve the cytoplasm content essential for the development of the embryo (Chaigne et al., 2012; Fabritius et al., 2011; McNally, 2013; Schuh and Ellenberg, 2008). This study reveals an unexpected role of ALADIN in spindle positioning at the oocyte cortex. Results indicate that ALADIN$^{-/-}$ oocytes fail to extrude their PB due to problems of spindle positioning and rotation. Meiotic spindle have to migrate from the centre to periphery of the oocyte. At the periphery, the spindle has to be oriented in a predetermined division plane to ensure that both daughter cells receive half of the chromosome content (Chaigne et al., 2012; Fabritius et al., 2011; McNally, 2013; Schuh and Ellenberg, 2008). It has been proposed that in somatic cells that astral microtubules generate pulling forces towards the cortex (McNally, 2013), which moves the spindle pole from the centre to the cell cortex. Oocyte spindles assemble in the absence of centrosome and have no apparent astral microtubules (McNally, 2013). In this model system, spindle-positioning is driven by a cytoplasmic actin network together with their regulated cofactors (Dumont et al., 2007a; Schuh and Ellenberg, 2008; Yi and Li, 2012). Here it was shown that ALADIN$^{-/-}$ oocyte spindles migrate slower and reach the cortex in a different orientation than WT oocytes; implying that ALADIN is a new regulator of spindle positioning. Nevertheless, no relation between ALADIN and actin has been published so far. However, ALADIN is localised around the spindle, where actin nucleation occurs and convection forces are generated (Bezanilla and Wadsworth, 2009; Grill et al., 2001).

A matrix assembles around the spindle, composed of nucleoporins, lamins and vesicles from Golgi and ER and has been proposed to function as a non-microtubule scaffold able to tether force generators and stabilise the mitotic spindle (Schweizer et al., 2014; Zheng, 2010). Studies in *Drosophila* showed that this structure is important for spatial and temporal control of mitosis (Schweizer et al., 2014). Due to the fact that oocyte meiotic maturation occurs in the absence of centrosomes, the presence of this structure, where ALADIN is located, may be crucial to generate non-microtubule forces for the migration and rotation of the spindle to interact properly with the

cortex. Further work is required to resolve ALADIN's role in spindle positioning and rotation in mouse oocyte maturation. Although this proposed model explains why ALADIN$^{-/-}$ oocytes fail to extrude their PBs, it does not explain why female mice are sterile. Nevertheless, the IVF experiments performed are a clear evidence of ALADIN's requirement for meiosis II and ultimately, mouse embryo development. ALADIN is not required for *Drosophila* embryo development, but when crossed with flies lacking centrosomes (also viable as a single mutant) they fail to develop (E. Griffis, unpublished observations). As in flies, the first mitotic divisions in mouse happen in the absence of centrosomes, suggesting that ALADIN, which while dispensable for mitosis in somatic cells, may be essential for mitosis in models lacking centrosomes.

Altogether, the present work shows that ALADIN plays a critical role in mouse oocyte meiosis with key roles in fundamental events for successful oocyte maturation, namely in spindle assembly, positioning and rotation, which concomitantly affect the robustness of oocyte maturation and impairs mouse embryo development.

5.Association of ALADIN with ciliary functions

5.1.Introduction

Cilia are membrane-bounded, centriole-derived, microtubule-containing projections from the cell surface. In interphase, centrioles can migrate to the cell surface, where the mother-centriole forms the basal body, the structure from where cilia start to assemble (Carvalho-Santos et al., 2011). Cilia are specialised sensory structures that are mechanical and chemosensitive. Their sensory function is converted into transduction cascades that participate in differentiation, migration and cell growth. Defects in ciliary proteins result in a multiplicity of diseases and syndromes termed ciliopathies (Bettencourt-Dias, 2011; Bettencourt-Dias, 2007; Gerdes, 2009). Abnormal biogenesis of cilia can also result in cancer or developmental problems. (Gerdes et al., 2009; Pazour and Witman, 2003). This may be due to the fact that numerous proteins involved in regulation of cell cycle are also involved in ciliogenesis. (Bettencourt-Dias and Glover, 2007).

Molecular traffic between the cytoplasm and the nucleus is controlled by nuclear pore complexes (NPCs), which are composed of approximately 30 different nucleoporins (NUPs). Recently, several NUPs have been found at the base of primary cilia (Kee et al., 2012; Kee and Verhey, 2013). Similar with their role at the NPC, it is proposed that these NUPs form a selective exclusion barrier at the base of the cilia where they concentrate certain proteins within the cilia but exclude others (Kee et al., 2012; Kee and Verhey, 2013; Obado and Rout, 2012). Therefore, it was examined whether ALADIN is a new NUP at this barrier. Here it has been identified the association of ALADIN with the cilium and it appears to control ciliary length and maintenance in the context of its associated syndrome.

5.2. ALADIN localises at the cilium

Recent studies have shown the localisation of several NUPs at the base of primary cilia (Kee et al., 2012). To test whether ALADIN also localises at cilia, primary cilia formation was induced by serum starvation of human fibroblasts. Cells were fixed and stained for a marker of cilia and a specific antibody for ALADIN. Although ALADIN is barely seen at the nuclear envelope due to the fixation method used (methanol); ALADIN seems to localises at the base of primary cilia, similar to other NUPs (Figure 34A).

Several brain development-related diseases have been linked to defects in the ciliary structure and motility (Nigg, 2009). Patients with triple A syndrome frequently suffer from neurological disturbances, which include global autonomic disturbance and microcephalies (Huebner et al., 2000). Therefore, the localisation of ALADIN was also tested in cultured fibroblasts from patients homozygously expressing the p.S263P and p.Q387X mutant forms of ALADIN (Kind, 2010). After 48 h of serum starvation, both mutants were observed to localise at the base of the cilia. Additionally, both proteins seem to be enriched at the base when compared with wild type conditions (Figure 34B and C).

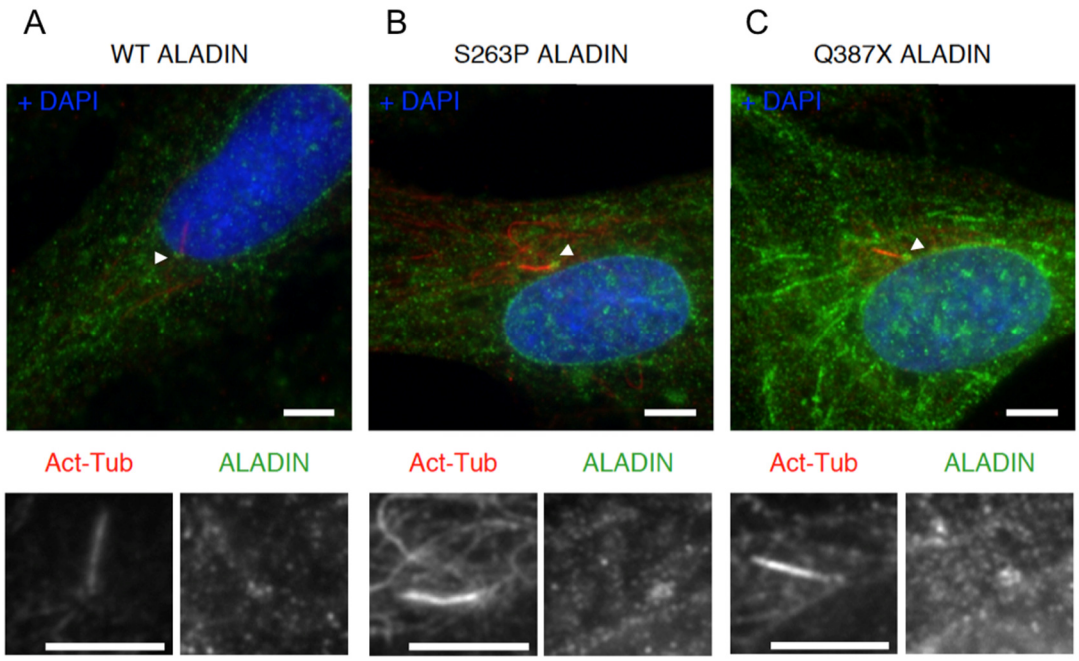


Figure 34: ALADIN localisation on cilia in fibroblast from triple A patients.
Representative images showing (A) wild type ALADIN localisation at the base of the cilium, as well as both mutant forms, S263P ALADIN (B) and Q387X ALADIN (C). See arrowhead. Magnified views are shown on the bottom panel for acetylated tubulin (left) and ALADIN (right) in all conditions tested. Scale bars, 5 µm.

5.3.Expression of ALADIN mutants affect cilia length in cells from patients with triple A syndrome

According to the cell type studied, cilia have a normal length range and, minimal deviations of this length are sufficient to produce pathogenic phenotypes (Broekhuis et al., 2013). Hence, it was examined if mutants of ALADIN affect the development and morphology of cilia. WT and ALADIN mutant fibroblast cell lines were serum starved for 24 or 48 h and the cilium length was measured. After 24 h, cilia were present in all conditions tested but both mutant forms of ALADIN had a 20% shorter cilium when compared with wild type conditions (Figure 35A). After 48 h, shorter cilia were still present in the S263P mutant cell line (**$p<0.05$) but not in the Q387X mutant form (Figure 35B).

Next, it was assessed the role of ALADIN in ciliary maintenance by measuring the number of ciliated cells. WT cells, serum-starved for 48 h, showed 48 ciliated cells in a total of 52 counted cells. In the case of S263P ALADIN fibroblasts, it was observed that only 33 of 53 cells were ciliated (Figure 35C). Overall, these preliminary results suggest that the ALADIN mutant forms described in triple A patients, namely the mutant form S263P, participates in ciliary length maintenance and disassembly.

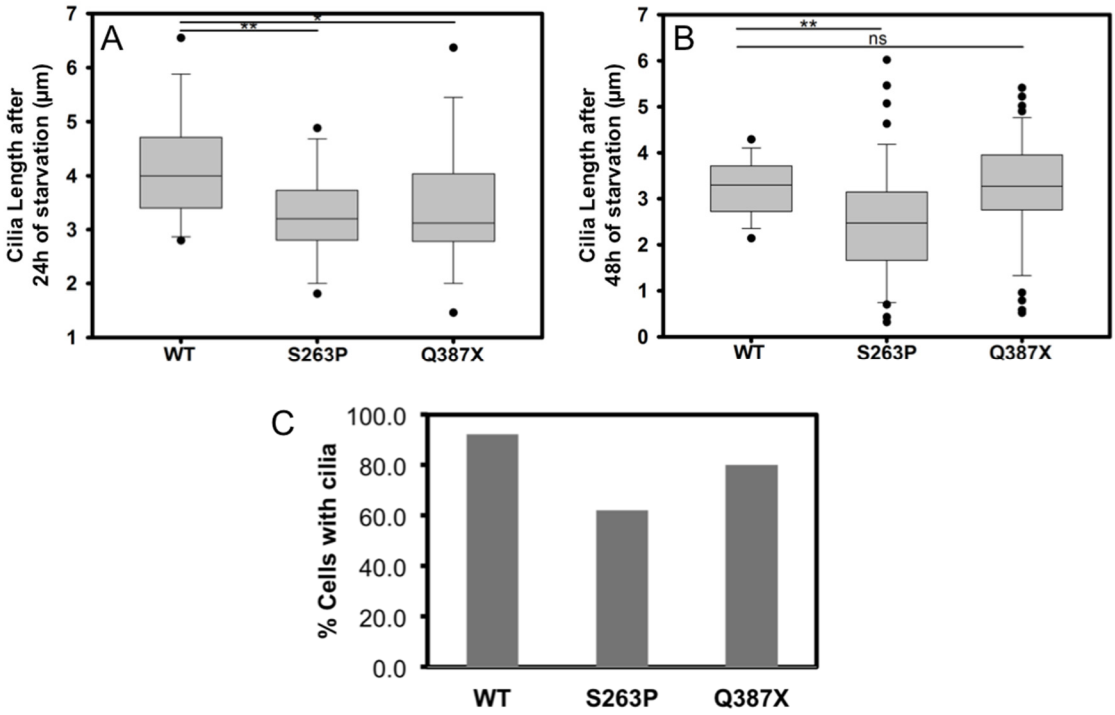


Figure 35: ALADIN is essential for ciliar length maintenance.
Fibroblasts were fixed and stained with acetylated-tubulin after serum starvation during 24 (A) or 48 (B) hours (h). Acetylated-tubulin was used to measure the cilia length. At least 15 cells were analysed in each condition with the exception of both mutant forms (S263P and Q387X) after 48 h of serum starvation (more than 45 cells), n=1 for 24 h and n=2 for 48 h. (C) Number of ciliated cells in S263P and Q387X mutant forms of ALADIN. WT ALADIN fibroblast cell line was used as control. n=1, at least 50 cell were counted in each condition. Cells were serum-starved for 48 h, then fixed and labelled for acetylated-tubulin, which was used as cilia marker. Box-and-whisker plot: middle line shows the median value; the bottom and top of the box show the lower and upper quartiles (25-75%); whiskers extend to 10th and 90th percentiles, and all outliers are shown. ns= no significant; *p<0.1 and **p<0.05.

5.4.Discussion

Recently several NUPs have been identified at the base of the cilium. Similar with their functions in nucleocytoplasmic transport, it was suggested that they form a selective barrier, excluding cargoes from or stimulating import into cilia (Kee et al., 2012; Obado and Rout, 2012). Here it has been identified the localisation of a new protein at the cilium, ALADIN. In agreement with these findings, another laboratory had also identified ALADIN at the cilium (L. Pelletier, personal communication), which supports the results here described.

ALADIN's function at the NPC is not been fully understood (Cronshaw and Matunis, 2004), making the presence of ALADIN at the base of the cilium difficult to interpret. Nevertheless, the expression of the S263P ALADIN mutant protein seems to produce cells with fewer and shorter cilia, which suggests a role of ALADIN in ciliary assembly.

Aurora A activation is necessary and sufficient to induce ciliary assembly and several proteins have been identified in the regulation of this pathway (Hubbert et al., 2002; Plotnikova et al., 2012; Pugacheva et al., 2007). Considering that ALADIN levels can influence the localisation of active Aurora A at the centrosome during mitosis (Chapter 3), one possible hypothesis is that ALADIN also influences this kinase's localisation at cilia. Therefore, further work is necessary to understand whether ALADIN's function in cilia is similar to its mitotic role. For instance, transient activation of Aurora A promotes ciliary resorption (Pugacheva et al., 2007) and by hypothesis ALADIN could also contribute to a redistribution of Aurora A at the cilium base and consequently dis-regulate cilia disassembly.

During interphase S263P and Q387X ALADIN mutant forms localise predominantly at the cytoplasm (Krumbholz et al., 2006). Hence, it would be interesting to details the localisation of WT ALADIN and their mutant forms at the cilium using quantitative immunofluorescence and understand if increased/decreased of ALADIN levels could affect ALADIN's cilia function.

Numerous disorders, such as ciliopathies and brain development-related diseases have been linked with ciliary dysfunctions and centrosome abnormalities (Nigg, 2009; Bettencourt-Dias,

2011). Mutations in ALADIN cause triple A syndrome but no definitive molecular mechanism behind the clinical phenotypes have been proposed (Huebner et al., 2000; Sarathi and Shah, 2010). Interestingly, S263P and Q387X ALADIN mutations have some of the mitotic phenotypes observed after ALADIN depletion, raising the possibility that mitotic errors may underlie the etiology of this syndrome. So, it would be also very interesting to further investigate ALADIN's function at cilia, namely in a brain development model, as patients with this syndrome usually have abnormal automatic nervous system development.

6.General Discussion and Future Directions

Nucleoporins (NUPs) have been the subject of great interest in recent years, and it is now accepted that they have roles during cell division (Chatel and Fahrenkrog, 2011; Gigliotti et al., 1998; VanGompel et al., 2015) and in cilia (Kee et al., 2012; Kee and Verhey, 2013). Furthermore, several NUPs have been associated with different diseases (Cronshaw and Matunis, 2004), increasing the attention on them and their roles in the cell. Triple A syndrome is a complex and multisystemic autosomal recessive disease associated with ALADIN (Tullio-Pelet et al., 2000). ALADIN is a NUP with related-functions in oxidative stress response, RNA import and steroidogenesis (Juhlen et al., 2015; Kind, 2010; Kind et al., 2009; Kiriyama et al., 2008). Nevertheless, the molecular mechanisms behind ALADIN's function or how ALADIN mutations cause the symptoms observed in patients with triple A syndrome remains unclear (Huebner et al., 2000; Prasad et al., 2014; Sarathi and Shah, 2010).

The aim of this thesis was to investigate the function of ALADIN in cell division, in particular in human mitosis and mouse oocyte meiosis. It was also sought to determine if ALADIN's role in mitosis could be related with triple A syndrome. Finally, preliminary results were obtained suggesting that ALADIN could be related with ciliary functions in the context of triple A syndrome.

6.1. ALADIN and cell division

Accurate chromosome segregation in each cell cycle is controlled by a sequence of spatially and temporally regulated events. After nuclear envelope breakdown (NEBD), the spindle is assembled in a synchronised manner. During NEBD, NPCs disassemble, and some of its protein components gain new functions (Chatel and Fahrenkrog, 2011). Here it is revealed that the NUP ALADIN also participates in mitosis and meiosis. Depletion of ALADIN during mitosis in human cells perturbs spindle assembly. Moreover, depletion of or overexpression of ALADIN spatially modifies the distribution of active Aurora A kinase within the centrosomes. In mouse oocyte meiosis, lack of ALADIN ($ALADIN^{-/-}$) also slows down the bipolar spindle assembly and affects its migration to the oocyte cortex, impairing polar body extrusion. Additionally, levels of ALADIN in mouse oocytes control the success of GVBD. These studies have exposed related but wide-ranging roles of ALADIN in multiple signaling pathways during cell division.

Cilia are also essential for regulating multiple signaling pathways (Plotnikova et al., 2009). Proteins in the cilia have been shown to be involved in cell cycle regulation, raising the possibility that cilia can control the entry and progression of the cell cycle (Plotnikova et al., 2009; Quarmby and Parker, 2005). Results here presented demonstrated that ALADIN is localised at cilia and ALADIN mutations affect ciliary maintenance; suggesting that ALADIN can act both in the cilium and in cell cycle regulation.

As a protein, ALADIN is a member of the WD domain family (Tullio-Pelet et al., 2000). This family of proteins is characterised by the presence of dipeptides repeats of tryptophan (W) and aspartate (D). WD domain family members regulate several biological processes such as signal transduction, gene expression regulation, genome stability and cell cycle control. (Xu and Min, 2011; Zhang and Zhang, 2015). WD-repeat domains form β-propeller structures that provides a platform for reversible protein-protein or protein-DNA interactions (Smith, 2000). It would be interesting to study how the surfaces of the WD-repeat domains of ALADIN are necessary for its cell division functions and which surfaces of the protein are making contacts with its binding partners.

On the other hand, the role of many proteins can be modulated by post-translational modifications such as phosphorylation (Royle, 2013). Many cell division events are coordinated and regulated by a complex network of cell cycle kinases, which regulate hundreds of different substrates to modify their activities and localisation (Nigg, 2001). An enormous effort has been dedicated to identifying these substrates and to understand the effects of their phosphorylation (Dephoure et al., 2008). Phosphoproteomic analysis of HeLa cells at various stages in the cell cycle identified ALADIN as one of the several proteins to be phosphorylated during mitosis (Olsen et al., 2010). Also, findings from the laboratory have shown that human ALADIN immunoprecipitated from human cells can be weakly phosphorylated *in vitro* by Aurora A kinase (T. Kasciukovic, unpublished data). Aurora A is an essential kinase controlling cell division (Lioutas and Vernos, 2014). This finding, suggests that ALADIN's function can be modulated by Aurora A during the cell cycle. In agreement with this idea, it was shown also that ALADIN's localisation during metaphase is sensitive to Aurora A kinase activity. During metaphase, ALADIN is localised around to the spindle pole, but when cells are treated with MLN8237 to specifically inhibit Aurora A kinase, ALADIN becomes more enriched at the spindle pole. Although it is not clearly understood how ALADIN's localisation affects its mitotic function, the relation between ALADIN's localisation, its regulation by Aurora A activity, and its reciprocal function in regulating Aurora A localisation, suggests that ALADIN's localisation is essential for its function.

In meiosis, ALADIN also localises around the spindle. Thus, additional analyses are necessary to determine whether ALADIN's localisation in mouse oocytes is also sensitive to Aurora A activity.

6.2. ALADIN as a regulator of Aurora A

A cell undergoes dramatic changes to ensure proper chromosome segregation into each daughter cell. To control such reorganisation of the cell, a complex network of mitotic kinases regulate hundreds of different substrates, modifying their activities and localisation (Nigg, 2001). One of these is the Aurora A kinase that resides at centrosomes and spindle poles (Barr and Gergely, 2007). Furthermore, Aurora A is required at mitotic entry, centrosome maturation, bipolar spindle assembly, and anaphase microtubule stability (Asteriti et al., 2011; Barr and Gergely, 2007; Lioutas and Vernos, 2014). This serine/threonine kinase is highly regulated by phosphorylation in a cell cycle dependent manner. After depletion or overexpression of ALADIN, Aurora A catalytic activity, measured by phosphorylation on Threonine 288, remains unaffected; however its localisation at the centrosome was significantly reduced. Additionally, ALADIN depletion only affected the spatial distribution of a subset of Aurora A substrates (NuMA, HURP, and Augmin), but not others (Plk1, Kif2a, Tacc3, Tpx2, NEDD1). These results demonstrate that Aurora A requires precise spatiotemporal regulation to properly modulate it substrates and that there are multiple and separable pathways by which Aurora A can regulate its substrates.

One of the most striking phenotype observed after ALADIN depletion was a profound perturbation in NuMA localisation. There is an intriguing interaction between NuMA and the nucleoporin Nup188, which is also required for the proper localisation of NuMA at the spindle poles (Itoh et al., 2013). Therefore, it cannot be rule out that Aurora A also regulates Nup188 and that the NuMA phenotype observed is downstream of a Nup188 misregulation, but given that Aurora A can interact with ALADIN and that overexpression and depletion of ALADIN both perturb the distribution of active Aurora A, it is favour the model in which ALADIN regulates NuMA through its effects on Aurora A.

In additional to spatial regulation of Aurora A phosphorylation, this kinase is also regulated by many protein-protein interactions with cofactors (Lioutas and Vernos, 2014). Tpx2 is known to be required for target and activity of Aurora A kinase onto the spindle and CEP192 onto the centrosome (Bayliss et al., 2003; Joukov et al., 2010; Joukov et al., 2014; Kufer et al., 2002).

While ALADIN depletion does not affect Tpx2 localisation within the spindle, CEP192 levels are reduced at the centrosome. Additionally, Aurora A and CEP192 can be imunoprecipitated with ALADIN in cells when Aurora A kinase is inactive (after MLN8237 treatment). Hence, it can be speculated that ALADIN controls the spatial localisation of Aurora A at the centrosome via CEP192. These proteins could form a ternary complex required for the proper targeting of Aurora A kinase. To further develop this hypothesis complementary studies are necessary, namely to confirm these interactions and find other relevant mitotic protein interactors of ALADIN mass spectrometry analysis can be used in cells arrested in mitosis.

In the work here presented was investigated the reciprocal regulatory relationship between Aurora A and ALADIN in mitotic human cells. However, it would be very interesting to investigate this regulatory pathway in other model systems. For instance, here it was described in mouse oocytes that perturbing ALADIN levels in mouse oocytes alters the timing and execution of germinal vesicular breakdown (GVBD), a marker of meiosis resumption. Two different studies have reported that Aurora A kinase is involved in the triggering meiotic resumption (Doyle et al., 2014; Solc et al., 2012). Additionally, they have shown that reduced levels of Aurora A delayed progression through meiosis I, and produces shorter spindles at metaphase I. Likewise, the absence of ALADIN in mouse oocytes slows the formation of the spindle, and the resulting spindles are shorter as described in Chapter 4 of this thesis.

Moreover, preliminary results on ALADIN's function in cilia suggest that ALADIN could have a role in cilia disassembly and length maintenance. Aurora A activation is necessary and sufficient to induce ciliary disassembly and several proteins have been identified in the regulation of this pathway (Hubbert et al., 2002; Plotnikova et al., 2012; Pugacheva et al., 2007). Considering that ALADIN levels can influence the localisation of active Aurora A at the centrosome during mitosis, one possible hypothesis is that ALADIN also influences this kinase's localisation at cilia. Therefore, further work is necessary to understand whether ALADIN's function in cilia is related with its mitotic role.

6.3.Cell division in triple A syndrome

ALADIN was originally discovered as a protein that when mutated caused triple A syndrome (Huebner et al., 2000; Tullio-Pelet et al., 2000). In subsequent studies, ALADIN was identified as a component of the NPC (Cronshaw et al., 2002) but no clear genotype-phenotype correlation of this syndrome has been identified so far (Sarathi and Shah, 2010). However, it has been proposed that alterations in reactive oxygen levels, steroidogenesis or defects in nucleotide excision repair produce some symptoms seen in patients who suffer from triple A syndrome (Juhlen et al., 2015; Kind, 2010; Kind et al., 2009; Kiriyama et al., 2008). Here it was described a novel function of ALADIN in mitosis. It was also shown that two mutations of ALADIN in triple A syndrome patients (S263P and Q387X) have similar phenotypes with cells where ALADIN has been silenced. These results indicate that ALADIN's role in mitosis can underline some of the symptoms observed in these patients.

The ALADIN mutant form Q387X produced a similar redistribution of the active Aurora A at the spindle poles as that observed after ALADIN siRNA treatment in HeLa cells. This mutant protein is a truncated form of ALADIN that lacks the C-terminus and its localisation seems not to be sensitive to MLN8237 treatment as the wild type ALADIN. From these results it can be concluded that ALADIN's localisation during metaphase and its role in the spatial regulation of Aurora A at the spindle pole requires the C-terminus of this protein. Curiously, the S436 amino acid, which it is absent in this ALADIN mutation, is predicted for be phosphorylated by Aurora A kinase (data not shown).

The expression of the other mutant form of ALADIN, S263P, also produces short spindles with a more disorganised metaphase plate and a reduction of active Aurora A at the centrosome, like Q387X expression and ALADIN depletion.

Here it was also shown that absence of ALADIN impairs mouse oocyte maturation and mouse development, leading to female fertility problems. Nevertheless, constitutive knock in of the mutant alleles S263P ALADIN protein into female mice rescues fertility problems, suggesting

that S263P mutant form acts differently from a complete absence of ALADIN in meiosis (A. Huebner, personal communication).

Finally, from the cilia studies here presented, the S263P ALADIN mutant form appears to affect cilia assembly and length regulation, which links triple A syndrome with ciliophaties.

The overall results presented here revealed unexpected relationships between triple A syndrome and cell division. Complementary studies are necessary to dissect and further understand this relation.

6.4. ALADIN in spindle assembly

The fundamental sequence of events in mitosis is the same as in meiosis (Ohkura, 2015). In both processes, ALADIN has a similar localisation at metaphase and its depletion or absence leads to a slower spindle assembly and shorter spindles. Results here presented suggest that absence of ALADIN impairs mouse oocyte maturation, which is critical for mouse development. Centrosomes facilitate and support the assembly of a bipolar spindle (Hinchcliffe, 2014), but they are absent from mouse oocytes and the first embryonic divisions (Ohkura, 2015). This could possibly explain why the deletion of ALADIN causes a more severe phenotype in mouse oocyte meiosis compared to somatic cells. In the absence of centrosome, ALADIN's function may become more essential. Hence, it would be interesting to investigate the role of ALADIN in the spindle assembly and cell division in models where centrosomes are absent or can be abolished. In support of this idea, experiments from the laboratory have found that ALADIN is not required for *Drosophila* embryo development, but when crossed to flies lacking centrosomes, which are also viable, embryos fail to develop (E. Griffis, and A. Müller, unpublished observations).

In *Drosophila* and human cells, it has been proposed that a spindle matrix facilitates the concentration and/or activation of spindle assembly regulators and potentially serves as a mechanical reinforcement for the structure (Schweizer et al., 2014; Zheng, 2010). This structure is formed by NUPs, lamins and vesicles derived from the Golgi and ER and localises around the spindle and at the spindle poles (Schweizer et al., 2014; Zheng, 2010). ALADIN's localisation and its role in spindle assembly are consistent with the proposed nature of a matrix component. Given that this protein has a conserved localisation during mouse oocyte maturation, it can be speculate that a similar structure exists and is potentially even more important in female meiosis and early embryonic divisions.

6.5. ALADIN in the context of cell polarity

Cell polarity and asymmetry play a fundamental role in stem cells and embryo development. Mouse oocytes undergo very asymmetrical divisions to keep the cytoplasmic material essential for fertilisation (Brunet and Maro, 2007; McNally, 2013). After GVBD (germinal vesicle breakdown) near or at the centre of the oocyte, the spindle starts to assemble. Then, it migrates to the cortex of the oocyte, where one set of homologous chromosomes is extruded into a polar body (PB). Coordinated cytoskeletal rearrangements are key to the development of oocyte polarity (migration and polar body extrusion). While spindle migration does not required microtubules (Verlhac et al., 2000), several reports have shown that the actin network and its regulators participate in the re-location of the spindle to the cortex (Dumont et al., 2007a; Schuh and Ellenberg, 2008; Verlhac et al., 2000; Yi and Li, 2012). Here it is described that ALADIN is a novel regulator of oocyte polarity, which participates in spindle re-localisation to the cortex. Results indicate that ALADIN$^{-/-}$ oocytes fail to extrude their PB due to problems in the spindle positioning and rotation when reaching the oocyte cortex. Further studies are required to explore whether ALADIN is required for the regulation of actin nucleation around the spindle. It would also be interesting to investigate whether cell polarity is impaired in early mouse development in fertilised knockout eggs.

Drosophila neuroblasts have a system where cortical polarity is induced by microtubules. In the absence of NuMA-related Mud protein, cortical polarity remains normal, however the metaphase spindle often fails to align with the cortical polarity axis (Siller et al., 2006). After ALADIN depletion in HeLa cells there was an increased of NuMA cortical levels compared with control cells. Therefore, it would be interesting to evaluate whether depletion of ALADIN in neuroblasts, namely at the NuMA cortical levels, would affect the cortical polarity axis.

Polarity also controls the orientation of cilia and ciliogenesis (Boutin et al., 2014). During interphase, centrosomes migrate to the cell periphery and mature into polar bodies, the base of the primary cilia. Given that ALADIN localises at the base of the cilium and ALADIN mutant lines have problems assembling cilia hint that ALADIN has a previously uncharacterised role in

ciliary assembly. This also raises the possibility that triple A syndrome may be a mild form of a ciliopathy. Additional studies are necessary to explore the importance of ALADIN in cell polarity, namely in neuronal function as patients with triple A syndrome usually have abnormal automomic nervous system development and microcephaly (Sarathi and Shah, 2010).

6.6. ALADIN and spindle forces

Forces generated by microtubules dynamics and mechanical properties within the spindle's proteins govern spindle assemble, maintenance and chromosome segregation (Civelekoglu-Scholey and Scholey, 2010; Dumont and Mitchison, 2009). Microtubules flux is not changed after ALADIN depletion in somatic cells. However, many of the phenotypes observed in human cells and mouse oocytes lacking ALADIN could be caused by unbalanced forces within the spindle. Although no forces within the spindle were measured in this work, it was observed an increased in kinetochore stretch, destabilisation of k-fibres, shorter spindles, and problems with chromosome alignment, which indicate that forces within the spindle are not properly regulated in the absence of ALADIN. It has been known for many years that there are forces acting on the spindle (Nicklas, 1983). Mitotic spindles reach a steady-state length at metaphase through the integrated action of molecular mechanisms that generate and respond to mechanical forces (Dumont and Mitchison, 2009). Such panoply of defects within the spindle following ALADIN's depletion cannot be fully explained, but these defects could be resulted from the improper recruitment of spindle assembly factors due to the redistribution of Aurora A localisation, which dramatically impacts force balancing within the spindle.

Increased kinetochore stretch and destabilised k-fibres within the spindle is an unusual phenotype from single depletion. Usually, it is thought that microtubule-kinetochore interactions stabilise k-fibres (King and Nicklas, 2000). Nevertheless, as observed after ALADIN depletion, depletion of MCRS1, which caps microtubule minus ends, produced a very similar phenotype of shorter, destabilised spindles with hyper-stretched kinetochores (Meunier and Vernos, 2011). However, unlike ALADIN, MCRS1 regulates microtubule flux during metaphase (Meunier and Vernos, 2011); suggesting that there are multiple ways in which tension within the spindle may be dis-regulated.

In mouse oocyte maturation, spindle-positioning is driven by a cytoplasmic actin network together with its regulating cofactors (Dumont et al., 2007a; Schuh and Ellenberg, 2008; Yi and Li, 2012). ALADIN is localised around the spindle, where actin nucleation occurs and convection

forces are generated (Bezanilla and Wadsworth, 2009; Grill et al., 2001). Finding that ALADIN$^{-/-}$ oocyte spindles migrate slower and reach the cortex in a different orientation than WT spindles implicate ALADIN as new regulator of spindle positioning possible by affecting external convection forces. However, there is also the possibility that other forces external to the spindle in mitosis and meiosis may also be perturbed when ALADIN is depleted.

Although it is not possible to say anything definitive about the existence or function of the spindle matrix, ALADIN localises around the spindle, during metaphase in both mitosis and oocyte meiosis, where this tensile matrix has been proposed to function (Zheng, 2010). Pickett-Heaps and collaborators showed that the ablation k-fibres in a half of a spindle leads to a compression of that side of the spindle where the severed pole is pushed towards the metaphase plate. They hypothesised that there must be an external non-microtubule-based compression force that is applied to the spindle and invoked the spindle matrix as the source of the force (Pickett-Heaps et al., 1997). Shortening of the spindle and destabilisation of the k-fibres could be a response to either a change in this external compression force or a loss of the ability of the spindle to resist it. Nonetheless, the data presented it this work suggests that destabilised k-fibres are caused by Aurora A re-distribution and the subsequent re-distribution of its substrates HURP and Augmin towards the spindle pole, and NuMA towards the centrosome.

While very little is known how forces are integrated during cell division, ALADIN's phenotype in mitosis and meiosis suggest its participation in the control of forces within the spindle.

7.Final remarks

Much of what it is known about ALADIN function comes from studies in interphase, particularly on its role in oxidative stress and steroidogenesis in the context of triple A syndrome (Juhlen et al., 2015; Kiriyama et al., 2008). Currently understanding of how mutations of ALADIN cause the triple A syndrome is very limited (Cronshaw and Matunis, 2004; Huebner et al., 2000; Prasad et al., 2014; Sarathi and Shah, 2010). With the data presented in this thesis, the functional roles of ALADIN were expanded in the context of cell division. Here it was demonstrated that ALADIN participates in mitosis, meiosis and ciliogenisis. In both mitosis and meiosis, ALADIN is important for proper spindle assembly. In mitosis, it was also discovered that ALADIN is a novel factor in the spatial regulation of the mitotic controller Aurora A kinase. Without ALADIN, active Aurora A spreads from centrosomes onto spindle microtubules, which affects the distribution of a subset of microtubule regulators and slows spindle assembly and chromosome alignment. ALADIN interacts with inactive Aurora A and is recruited to the spindle pole after Aurora A inhibition. Curiously, some of the mitotic phenotypes observed after ALADIN depletion also occurs in cells from triple A syndrome patients, which raises the possibility that mitotic errors may underlie the etiology of this syndrome. In meiosis, ALADIN contributes to trigger the resumption of mouse female meiosis. Homozygous knockout of ALADIN in mouse oocytes was shown to slow bipolar spindle assembly and migration of the spindle to the cell cortex. It was also observed that polar body extrusion was impaired due to problems in the spindle rotation before anaphase. ALADIN is necessary for proper oocyte maturation and is critical for early embryonic development.

Before cell division begins, primary cilia can be assembled during interphase (Pazour and Witman, 2003). At this stage ALADIN is present at the NPC that is embedded at the nuclear envelope (Cronshaw et al., 2002). Results in this work revealed that ALADIN at this stage also localises at the base of primary cilia, and it may have a role in the maintenance and/or disassembly of the cilia.

These findings significantly advance the general understanding of ALADIN's function in the cell. However, many important questions regarding the regulation of ALADIN and its function across cell division still remain unanswered. For example, further studies are necessary to explore how the phenotypes observed in patients with triple A syndrome are related with ALADIN's function in cilia, mitosis, meiosis and development.

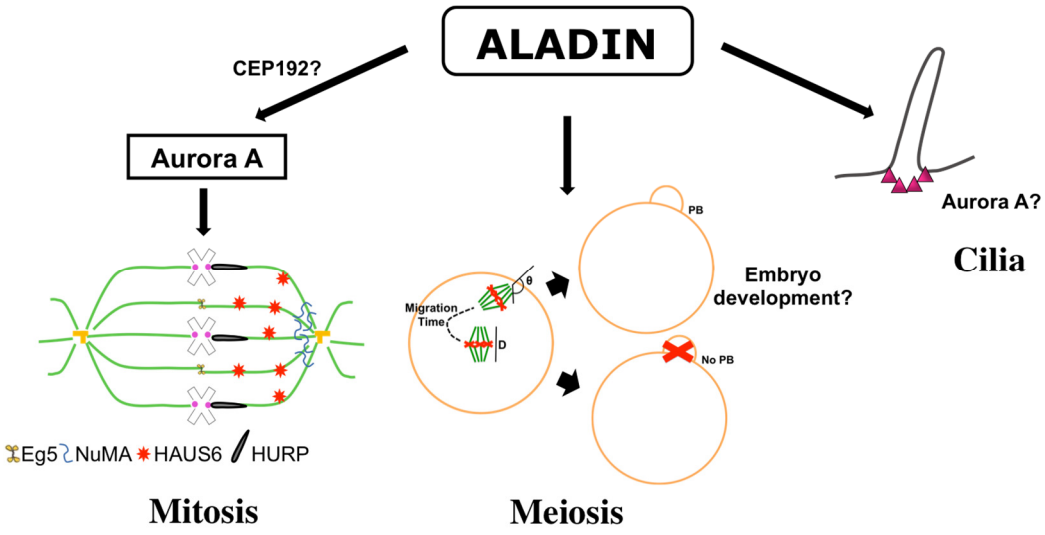


Figure 36: Overall diagram of the main findings presented in this thesis.
ALADIN is a novel nucleoporin involved in mitosis that spatially regulates Aurora A, possibly through CEP192. After depletion of ALADIN in human cells, active Aurora A spreads from centrosomes onto spindle microtubules, which affects the distribution of a subset of spindle assembly factors (Eg5, NuMA, HAUS6 and HURP) producing a less robust mitotic spindle. Similarly to what happens in mitosis, ALADIN also participates in meiotic mouse oocyte maturation. Impairment of ALADIN slows spindle assembly, migration and reduces oocytes ability to extrude polar bodies during meiosis I, which concomitantly also affects the robustness of oocyte maturation. Lack of ALADIN impairs mouse embryo development maybe due to problems in the female meiosis process. ALADIN was found to locate at the base of the cilia (pink triangles), and this work suggests its participation in cilia disassemble. Further studies are necessary to test whether ALADIN also regulates Aurora A in cilia.

8.Appendix

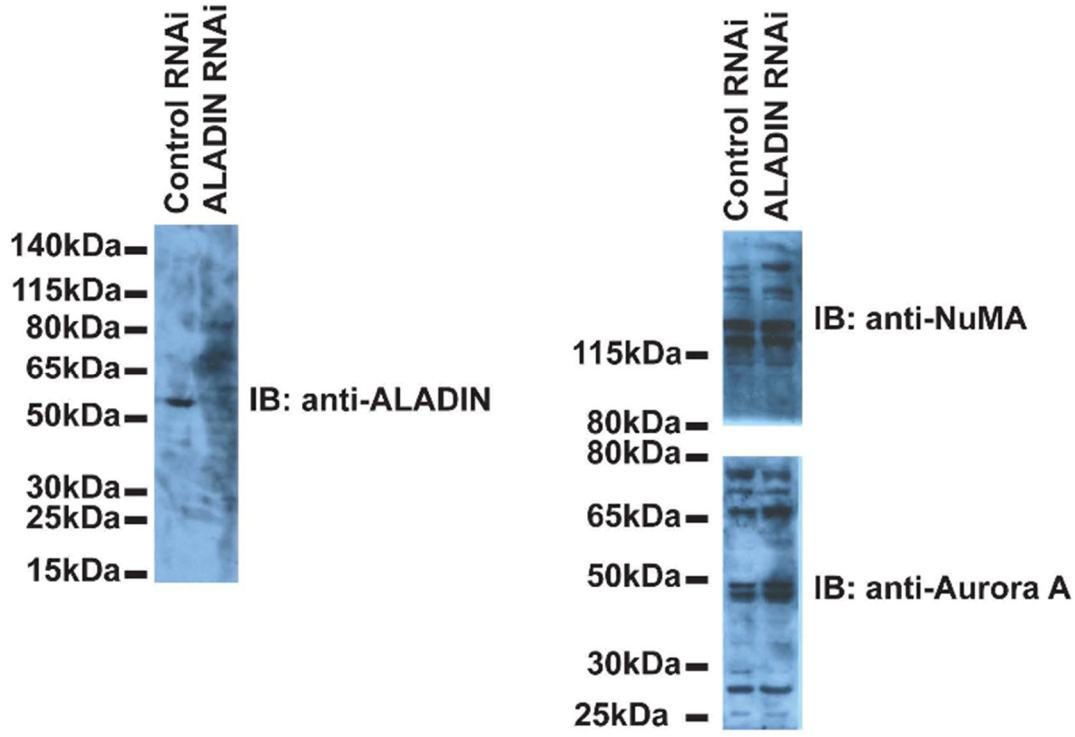


Figure 37: ALADIN depletion in HeLa cells does not change protein levels of Aurora A and NuMA. Whole cell lysates from cells treated with control and ALADIN specific siRNAs were probed with an anti-ALADIN antibody before being cut, stripped, and probed with anti-NuMA and anti-Aurora A antibodies.

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